CHAPTER V, PART A: Ethane Formation from Chloroplasts Exposed to SO_2 - A Measure of Lipid Peroxidation

1. Introduction

Sulfur dioxide is a major air pollutant causing damage to plants. The increasing demand for the use of coal for power generation may lead to an increase in SO_2 pollution (Rubin, 1981). Various physiological parameters are affected in plants exposed to SO_2. These include inhibition of photosynthesis and growth rate which can occur without visible injury (Hallgren, 1978). One of the first ultrastructural changes observed in plants exposed to SO_2 is damage to chloroplast membranes (Fischer et al., 1973; Hallgren, 1978; Wellburn et al., 1972), resulting in a loss of membrane integrity, which is vital to all processes in the plant. Proteins are susceptible to attack by sulfite (Schroeter, 1966) which would lead to altered membrane structure and function. Recent work from our laboratory (Lizada and Yang, 1981) has shown that sulfite can induce the *in vitro* peroxidation of linoleic and linolenic acid which could lead to the alteration of membranes. Inasmuch as these two fatty acids comprise approximately 75% of those found in chloroplast membranes, peroxidation of these fatty acids may be an important factor contributing to damage *in vivo*.

Sulfite can undergo very rapid oxidation to sulfate through a free radical mechanism which predominates at low concentrations (Abel, 1951; Schroeter, 1966). Free radicals produced during the oxidation of sulfite have been reported to affect the *in vitro* destruction of methionine and tryptophan (Yang, 1970; Yang, 1973) indole-3-acetic acid (Yang and Saleh, 1973), chlorophyll (Peiser and Yang, 1977), β-carotene (Peiser and Yang, 1979), and oxidized NADH and NADPH (Tuazon and Johnson, 1977). Also the peroxidation of linolenic and linoleic acids has been attributed to free radicals produced during sulfite oxidation (Lizada and Yang, 1981).

Because chloroplasts are rich in linoleic and linolenic acid and appear to be an early site of damage by SO_2, we have studied whether sulfite could induce lipid peroxidation in chloroplasts via free radical mechanisms.

2. Materials and Methods

Chloroplasts were isolated from spinach (Spinacia oleracea L.) obtained from a local grower or from a market. Leaves were passed through a juice extractor (Acme Supreme) along with isolation medium containing 50 mM phosphate buffer (pH 7.8), 0.33 M sorbitol and 2 mM $MgCl_2$ at 0°C. Chloroplasts were passed through Miracloth and then centrifuged 2 min at 1,700g. The pellet was resuspended in incubation medium (same as the isolation medium except that 100 mM glycylglycine [pH 7.8] was used instead of phosphate buffer) and then layered on top of Percoll medium (40% [v/v] Percoll in incubation media). This was centrifuged in a swinging bucket rotor for 5 min at 3,000g. Broken chloroplasts remained at the buffer-Percoll interface while intact chloroplasts penetrated the Percoll. Intactness was 80% or greater as estimated by the ferricyanide method (Lilley et al., 1975). Freeze-treated chloroplasts were prepared by placing broken chloroplasts at -10°C overnight.

Lipid peroxidation from chloroplasts was determined by measuring ethane formation (Dillard and Tappel, 1979; Dumelin and Tappel, 1977). A standard reaction mixture containing chloroplasts (400 µg Chl) and 0.1 µmol $FeCl_3$ in 1 ml incubation medium was incubated in a 10-ml Erlenmeyer flask, which was sealed with a serum stopper and gently shaken at 25°C over a 15 w cool-white fluorescent lamp which provided 200 $\mu E\ m^{-2}\ s^{-1}$ of illumination. Seven µl of solution containing 1.4 mol Na_2SO_3 plus 0.7 nmol EDTA or 1.4 µmol Na_2SO_4 plus 0.7 nmol EDTA in controls were added every 3 min using a syringe with hypodermic needle. EDTA was included in the sulfite solution to prevent autooxidation. At various times, the gas headspace was sampled and injected into a gas chromatograph equipped with an alumina column for ethane measurement.

Sulfite oxidation was measured both by sulfite determination using the pararosaniline method (Scaringelli et al., 1967) and by measuring O_2 uptake. O_2 uptake was determined in a 3 ml reaction mixture containing 200 µg Chl with a Clark O_2 electrode under darkness or under 1,500 $\mu E\ m^{-2}\ s^{-1}$ of red illumination.

3. Results

Ethane, derived from the decomposition of the 16-hydroperoxide of linolenic acid, has been used in many systems as a measure of lipid peroxidation (Dillard and Tappel, 1979; Dumelin and Tappel, 1977; Riely et al., 1974). In broken and intact chloroplasts sulfite greatly stimulated ethane production compared with sulfate (sulfate was always added to controls) (Table V.1). Sulfite caused approximately a 10-fold increase in the ethane production in intact chloroplasts as compared to the sulfate control. The effect of sulfite, however, was even more marked in broken chloroplasts. Ethane production from freeze-treated broken chloroplasts produced the greatest amounts of ethane (Table V.1).

Differences in sulfite-induced ethane formation were consistently observed with chloroplasts isolated from spinach grown in different seasons. Chloroplasts from winter spinach produced more ethane (800-900 pmol/h) than those from spring spinach (300-400 pmol/h). The effects of certain metals were examined to determine if ethane formation could be increased in chloroplasts from spring spinach. Ethane formation was enhanced by the addition of 100 μM $FeCl_3$ and greatly reduced by 1 mM EDTA (Fig. V.1). $FeSO_4$ was as effective as $FeCl_3$, whereas $CuCl_2$ (100 μM) had no effect upon ethane production. Whether an inhibitor was present or lower concentrations of stimulators (such as metal ions) were present in spring compared to winter spinach was not examined. When $FeCl_3$ was deleted, less ethane was formed, although no qualitative changes were observed. In some systems (Kochi, 1967), cupric or cuprous ion modifies the ratio of ethylene to ethane produced from linolenic acid, but in our system it had little effect upon this ratio. Ethylene production was usually only 3% or less of the ethane production.

Light was very important for sulfite-induced ethane formation. In the dark only about 10 to 20 pmol ethane was produced after 1 hr in the presence or absence of sulfite with or without $FeCl_3$ (Fig. V.1). $MnCl_2$ was not added to the isolation or incubation medium because it promoted ethane production in the dark with sulfite. In several systems Mn^{2+} has been used to initiate the free radical oxidation of

sulfite (Peiser and Yang, 1977; Yang, 1970, 1973; Yang and Saleh, 1973).

Sulfite oxidation, measured by sulfite loss, occurred throughout the incubation period although complete oxidation did not occur (Fig. V.2). The amount of sulfite added, 1.4 μmol every 3 min, was necessary because a 25% reduction in sulfite concentration caused a 70% reduction in ethane formation. One explanation for these results is that a portion of the sulfite reacted with some chloroplast component forming a complex or adduct which was stable against oxidation, yet it would react with pararosaniline reagent in the sulfite assay. In the dark, little sulfite was oxidized with or without 100 μM $FeCl_3$ (data not shown). When sulfite oxidation was measured using the O_2 electrode, the O_2 uptake rate in dark was approximately 24 nmol/min, whereas that in the light was approximately 240 nmol/min, measured in a 3-ml volume. These rates are similar to those obtained by Asada and Kiso (1973) examining sulfite oxidation in chloroplasts.

Inasmuch as light was necessary for sulfite-induced ethane production as well as sulfite oxidation, the effect of various photosynthetic electron transport modulators was examined (Table V.2). DCMU (3-[3,4-dichlorophenyl]-1,1-dimethylurea), which inhibits photosynthetic electron transport, as well as PMS (phenazine methosulfate), which promotes cyclic electron flow, inhibited both ethane production and sulfite oxidation (measured as O_2 uptake). These results demonstrate the dependence of sulfite oxidation and ethane formation upon photosynthetic electron transport and noncyclic electron flow. MV (methylviologen, paraquat), which facilitates the reduction of O_2 on the reducing side of PSI to form O_2^- (Dodge, 1977), greatly stimulated ethane production in the presence of sulfite, but caused a comparatively small amount of ethane formation in the absence of sulfite. Although we measured sulfite oxidation in the presence of MV with the oxygen electrode, this is complicated because oxygen uptake occurs with MV in the light in the absence of sulfite. Therefore, we determined sulfite oxidation in the presence of MV using the pararosaniline assay, under identical conditions as for ethane formation. Contrary to our expectation, MV had only a small effect

upon sulfite oxidation (Fig. V.2) in comparison with its effect upon ethane formation in the presence of sulfite (Table V.2).

The participation of free radicals was implicated by the effective inhibition of both sulfite oxidation and ethane formation by the radical scavengers, tiron and ascorbate (Table V.3). Tiron has been reported to be a specific scavenger for O_2^- (Greenstock and Miller, 1975), but recent evidence indicates that it also effectively scavenges hydroxyl radical (Bors et al., 1979). Ascorbate can likewise react with O_2^- and hydroxyl radical (Halliwell, 1981). Ethanol, a hydroxyl radical scavenger, caused a small amount of inhibition of ethane formation; formate, which converts hydroxyl radical to O_2^- (Behar et al., 1970), caused a small stimulation of ethane formation and sulfite oxidation. The participation of hydroxyl radical is further suggested from preliminary observations that when mannitol was used in place of sorbitol as osmoticum, an inhibition of ethane production was observed. Mannitol is a hydroxyl radical scavenger and approximately twice as effective as sorbitol in this respect (Asada and Kiso, 1973). Glycylglycine was specifically chosen as the buffer since Tricine and Hepes inhibited ethane formation. Asada and Kiso (1973) also reported that Tris and Tricine inhibited sulfite oxidation in illuminated chloroplasts.

The close interrelation between sulfite oxidation and ethane formation and their dependence upon photosynthetic electron transport is demonstrated in the above results. In the absence of photosynthetic electron transport in the dark (Fig. V.1) or upon its inhibition with DCMU and PMS (Table V.2), sulfite oxidation and ethane formation were inhibited. Also, the radical scavengers tiron and ascorbate effectively inhibited both sulfite oxidation and ethane formation.

Inasmuch as singlet oxygen has been reported to be involved in the peroxidation of chloroplast lipids (Shimazaki et al., 1980), we examined the effect of DABCO and sodium azide (Table V.3). DABCO (2,4-diazobicyclo-[2,2,2]-octane) has been used as a singlet oxygen scavenger, although recent evidence indicates it also can serve as an effective radical scavenger (Packer et al., 1981). Sodium azide is considered an effective quencher of singlet oxygen with a rate

constant of 2.2×10^8 M^{-1} s^{-1} at 0.5 mM (Hasty et al., 1972). Results from these two compounds (Table V.3) do not indicate the participation of singlet oxygen in the production of ethane in our system. Chloroplasts contain both the Cu, Zn, and the Mn forms of superoxide dismutase (Asada, 1980). The stimulation of ethane formation and sulfite oxidation with azide and cyanide (Table V.3) may result from their inhibition of Cu, Zn-superoxide dismutase thereby increasing the concentration of O_2^-. Additionally, azide and cyanide could stimulate sulfite oxidation by complexing with metals which might stimulate radical-mediated reactions (McCord and Day, 1978).

The specific involvement of O_2^- was indicated by inhibition of ethane formation with superoxide dismutase (Table V.4). The amount of superoxide dismutase added was equivalent to the amount of endogenous enzyme activity of intact chloroplasts as determined by the xanthine:xanthine oxidase assay. The lack of complete inhibition with superoxide dismutase suggests that either another radical in addition to O_2^- is involved or the site of O_2^- formation, presumably on the thylakoid membranes, is not easily accessible by the exogenously added superoxide dismutase; in contrast, this site might be more accessible to a much smaller molecule like tiron which renders it a more effective inhibitor. Although the small inhibition by catalase indicates that H_2O_2 was participating in ethane formation, in intact but not broken chloroplasts, an effective H_2O_2 scavenging system exists (Nakano and Asada, 1980) which presumably would greatly reduce the participation of H_2O_2 in intact leaves. Since BSA (bovine serum albumin) had no effect upon ethane formation, the effects of superoxide dismutase and catalase were catalytic and not simply a nonspecific protein effect.

In most of these experiments $FeCl_3$ was present along with a small amount of EDTA (0.7 nmol added every 3 min with SO_3^{2-}). Fe-EDTA can stimulate free radical reactions in some systems (McCord and Day, 1978). In our chloroplast system, however, comparable amounts of ethane were formed in the presence or absence of $FeCl_3$ and EDTA. The amount of inhibition of ethane formation by superoxide dismutase and stimulation by formate was not altered when $FeCl_3$ and EDTA were excluded. This indicates our results are not dependent upon an

Fe-EDTA complex, but does not exclude the involvement of an endogenous metal complex.

4. Discussion

The aerobic oxidation of sulfite can be initiated by UV light (Schroeter, 1966), metals (Abel, 1951; Yang, 1970), photosensitized dyes (Peiser and Yang, 1977) and enzymic reactions (Fridovich and Handler, 1961), all of which produce free radicals. The superoxide radical appears to be the radical responsible for initiation in some of these cases (Yang, 1970), and a scheme for this reaction has been proposed (Abel, 1951; Yang, 1970).

$$O_2^- + SO_3^{2-} + 2H^+ \longrightarrow SO^{3-} + 2OH \quad (1)$$

$$SO_3^- + O_2 \longrightarrow SO_3 + O_2^- \quad (2)$$

$$SO_3^{2-} + OH + H^+ \longrightarrow SO_3^- + H_2O \quad (3)$$

$$SO_3^- + OH + H^+ \longrightarrow SO_3 + H_2O \quad (4)$$

$$2SO_3^- \longrightarrow SO_3 + SO_3^{2-} \quad (5)$$

$$SO_3 + H_2O \longrightarrow SO_4^{2-} + 2H^+ \quad (6)$$

Sulfite oxidation is maintained by the propagation reactions (equations 1,2,3) with the production of O_2^-, OH and SO_3^-. The termination reactions (equations 4,5,6) lead to sulfate formation.

Asada and Kiso (1973) reported that photosynthetic electron transport or illuminated chloroplasts initiated the aerobic free radical oxidation of sulfite. Experimental evidence indicates that O_2 serves as an electron acceptor on the reducing side of PSI forming O_2^- (Asada, 1980; Goldbeck et al., 1980) which in turn initiates sulfite oxidation. Our results are similar to those reported by Asada and Kiso (1973), showing the dependence of sulfite oxidation on photosynthetic electron transport. They observed inhibition of sulfite oxidation by DCMU and radical scavengers as we observed (Tables V.2 and V.3). Our results link photosynthetic electron transport-initiated sulfite oxidation to the peroxidation of membrane lipids in chloroplasts, by which SO_2 damage to plants might be mediated. In each experiment where sulfite oxidation was either inhibited or stimulated, ethane formation responded likewise, except for the experiment with MV. MV increased ethane formation 6- to 7-fold over that caused by sulfite alone (Table V.2) but had little

effect upon sulfite oxidation (Fig. V.2). One possible explanation is that MV cation interacts with sulfite or products of sulfite oxidation producing a very reactive species which greatly enhances ethane formation without affecting sulfite oxidation.

Sulfite-induced lipid peroxidation has been reported in corn oil emulsions (Kaplan et al., 1975) and emulsions of linoleic acid and linolenic acid (Lizada and Yang, 1980). This peroxidation of linoleic and linolenic acids appeared to proceed by a free radical reaction according to the following scheme where LH represents linoleic or linolenic acid (Lizada and Yang, 1980):

$$SO_3^- + LH \longrightarrow LHSO_3^- \quad (7)$$

$$LHSO_3^- + HSO_3^- \longrightarrow LH_2SO_3 + SO_3^- \quad (8)$$

$$SO_3^- + LH \longrightarrow L + HSO_3^- \quad (9)$$

$$L + O_2 \longrightarrow LOO \quad (10)$$

$$LOO + LH \longrightarrow LOOH + L \quad (11)$$

The authors suggested that SO_3^-, which could result from equation 1 or 3, was the important radical which mediated the propagation steps via addition (equation 7) and hydrogen abstraction (equation 9). Hydrogen abstraction from polyunsaturated fatty acids is considered to be one of the primary steps in the free radical-mediated peroxidation of polyunsaturated fatty acids (Mead, 1976). Similarly, hydrogen abstraction from NADH by CO_3^- has been demonstrated during the sulfite-mediated oxidation of NADH to NAD (Tuazon and Johnson, 1977). Based on this information and our results, we propose that of the radicals formed during the O_2^- initiated sulfite oxidation (equations 1,2,3) SO_3^- is the primary radical causing the peroxidation of chloroplast lipids. OH appears to play only a minor role since ethanol and formate had small effects upon ethane formation (Table V.3). O_2^- alone does not appear to be important in ethane formation since MV, which facilitates O_2^- formation without sulfite, caused a relatively small amount of ethane formation (8%) as compared with that caused by sulfite (Table V.2).

However, we cannot exclude the possibility that lipid peroxidation results from an interaction of two or more radicals rather than only one radical. An alternative explanation for the enhanced ethane production from sulfite and MV is that this synergism

results from an interaction between O_2^-, at an increased concentration resulting from MV, and other radicals from sulfite oxidation. Kong and Davison (1980) have shown that interactions between oxy radicals could lead to greater amounts of membrane permeability in erythrocyte ghosts than was expected from the summed effects of the individual radicals.

An outline of our results is presented in Figure V.3. Photosynthetic electron transport provides electrons to reduce O_2 to O_2^- which initiates sulfite oxidation. Radicals produced from sulfite oxidation then lead to the peroxidation of membrane lipids resulting in the formation of ethane.

Further work is needed to determine whether this free radical mechanism of sulfite-induced lipid peroxidation plays an important role in the in vivo damage to plants exposed to SO_2. However, regardless of the specific mechanism, there is evidence from in vivo experiments implicating free radicals, specifically O_2^-, in SO_2 phytotoxicity. Tanaka and Sugahara (1980) have reported that young poplar leaves contained more superoxide dismutase activity and were more resistant to SO_2 injury than old leaves. Also, they observed that low levels of SO_2 could induce superoxide dismutase activity in leaves and these leaves were subsequently more resistant to injury by high levels of SO_2 than leaves without the prefumigation.

CHAPTER V, PART B: Ethylene Formation from Corn Exposed to SO_2

1. Introduction

Ethylene is produced by plants under normal conditions in relatively low amounts, but when plants are perturbed or injured, elevated production rates usually occur (Yang and Pratt, 1978). Stress- or wound-induced ethylene production has been reported for plants exposed to ozone (Craker, 1971; Tingey et al., 1976) and SO_2 (Peiser and Yang, 1979; Bressan et al., 1979; Tingey, 1980).

It has been suggested that ethylene formation may be a convenient indicator of stress (Tingey, 1980). With some forms of stress relatively large amounts of ethylene are formed without visible injury to the plant (Tingey, 1980), yet in each of the studies with SO_2 (Peiser and Yang, 1979; Bressan et al., 1979; Tingey, 1980) the levels used caused visible injury. This study was conducted to determine if ethylene is formed in response to levels of SO_2 that cause no visible injury and to evaluate the applicability of ethylene formation as an indicator of stress. We also wanted to examine if differential varietal sensitivity to SO_2 occurs among three corn cultivars which have been described as having differing ozone sensitivities.

2. Materials and Methods

Three corn cultivars were used which have been described to differ in their sensitivity to ozone. NC+59 (field variety) and Bonanza (sweet variety) have been shown to be ozone resistant and NK 51036 (sweet variety) has been described to be ozone sensitive. Two week old plants were fumigated for 6 hr with 0.5 or 0.3 $\mu\ell\ \ell^{-1}$ SO_2. A description of the fumigation system is given in Chapter I.

For ethylene measurement leaves were cut from plants immediately (0 hr), 2 hr and 5 hr following fumigation. Each sample contained three leaves (the third leaf from three plants) which were placed in 52 ml test tubes containing 0.5 ml water and samples were run in triplicate. The tubes were sealed with serum stoppers, kept in the dark at 25°C for 1 hr at which time a 1 ml gas sample was taken for ethylene measurement using a gas chromatograph with alumina column and flame-ionization detector.

3. Results

Ethylene formation following a 6 hr fumigation of 0.5 $\mu\ell\ \ell^{-1}$ SO_2 was measured in five experiments; the data from two of these experiments is shown in Table V.5. It should be noted that no visible damage occurred under these conditions. Of the three cultivars tested only NC+59 was found to show enhanced ethylene formation in response to this level of SO_2. This was observed in each of the five experiments. However, the amount of ethylene formed in NC+59 was relatively small, from 3-20 times that of control levels, and this usually occurred in a transient peak between 0 and 3 hr following the fumigation. In experiment 1 the peak in ethylene formation occurred approximately 2 hr following the fumigation but no increase in ethylene was observed at 0 or 5 hr. In experiment 2, the peak occurred immediately following the fumigation, at 0 hr.

Three fumigations were conducted using 0.3 $\mu\ell\ \ell^{-1}$ for 6 hr with NC+59 since this was the only cultivar which showed enhanced ethylene production at 0.5 $\mu\ell\ \ell^{-1}$ SO_2. In none of these experiments was there a clear enhancement in ethylene production as a result of the SO_2 fumigation.

4. Discussion

Corn cultivar NC+59 was the only cultivar that responded to six hours of 0.5 $\mu\ell\ \ell^{-1}$ SO_2 with an increase in ethylene production, suggesting that this cultivar is more sensitive to SO_2 than Bonanza or NK 51036. This conclusion is consistent with results obtained exposing corn seedling roots to SO_2. At the lowest exposures (0.3 to 0.5 $\mu\ell\ \ell^{-1}$ SO_2) NC+59 was the most adversely affected of the three cultivars (Chapter IV).

When plants were exposed to a single dose of 1.5 $\mu\ell\ \ell^{-1}$, NC+59 showed the greatest visible injury; at 0.5 $\mu\ell\ \ell^{-1}$ on four successive days, NC+59 had significantly decreased root and shoot fresh weight (Chapter IV).

Since visible injury does not occur after a six-hour exposure to 0.5 $\mu\ell\ \ell^{-1}$ SO_2 and since other results confirm that NC+59 is the most sensitive cultivar to respond to low levels of SO_2, ethylene is an indicator of SO_2 stress. For NC+59 the threshold of response seems to

be 0.5 $\mu\ell\ \ell^{-1}$ SO_2, producing a small and transient peak of ethylene. This threshold of response is different for each cultivar and species. For example, in work with alfalfa (Peiser and Yang, 1979) after exposure to 0.7 $\mu\ell\ \ell^{-1}$ SO_2 for seven hours large amounts of ethylene were produced for 20 hours after fumigation ended; threshold response may occur at levels well below 0.7 $\mu\ell\ \ell^{-1}$. Generally, ethylene levels increase as the stress increases (Yang and Pratt, 1978). Bressan et al. (1979) observed that cucumber plants produced increasing amounts of ethylene as exposure to SO_2 increased. Although an ethylene response to SO_2 levels higher than 0.5 $\mu\ell\ \ell^{-1}$ has not been plotted for these corn cultivars, the response threshold for a single six-hour exposure has been observed and differences in cultivar response have been found to correlate with cultivar sensitivity to SO_2.

Summary

Ethane formation, as a measure of lipid peroxidation, was studied in spinach (Spinacia oleracea L.) chloroplasts exposed to sulfite. Ethane formation required sulfite and light, and occurred with concomitant oxidation of sulfite to sulfate. In the dark, both ethane formation and sulfite oxidation were inhibited. Ethane formation was stimulated by ferric or ferrous ions and inhibited by ethylenediamine tetraacetate. The photosynthetic electron transport modulators, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and phenazine methosulfate, inhibited both sulfite oxidation and ethane formation. Methyl viologen greatly stimulated ethane formation, but had little effect on sulfite oxidation. Methyl viologen, in the absence of sulfite, caused only a small amount of ethane formation in comparison to that produced with sulfite alone. Sulfite oxidation and ethane formation were effectively inhibited by the radical scavengers, 1,2-dihydroxybenzene-3,5disulfonic acid and ascorbate. Ethanol, a hydroxyl radical scavenger, inhibited ethane formation only to a small degree; formate, which converts hydroxyl radical to superoxide radical, caused a small stimulation in both sulfite oxidation and ethane formation. Superoxide dismutase inhibited ethane formation by 50% when added at a concentration equivalent to that of the endogenous activity. Singlet oxygen did not appear to play a role in ethane formation, inasmuch as the singlet oxygen scavengers, sodium azide and 1,4-diazobicyclo-[2,2,2]-octane, were not inhibitory. These data are consistent with the view that O_2 is reduced by the photosynthetic electron transport system to superoxide anion, which in turn initiates the free radical oxidation of sulfite, and the free radicals produced during sulfite oxidation were responsible for the peroxidation of membrane lipids, resulting in the formation of ethane.

Low levels of ethylene are normally produced by plants, but when a plant is injured ethylene production increases. It has therefore been suggested that ethylene formation is a stress indicator and that increasing levels of production indicate increasing levels of stress. This study was undertaken to determine ethylene response to low levels of SO_2 that cause no visible injury and to determine differential ethylene responses between cultivars. Three corn cultivars were

chosen because of their differential response to ozone and SO_2 (Chapter IV). Two week old corn plants were fumigated six hours with 0.3 and 0.5 $\mu\ell\ \ell^{-1}$ SO_2. Leaves were removed from the plants 0, 2 and 5 hours following termination of exposure, and ethylene production was measured. No increase in ethylene formation was observed in any of the cultivars at 0.3 $\mu\ell\ \ell^{-1}$ SO_2. At 0.5 ppm only NC+59 responded with increasing ethylene production. Previous research (Chapter IV) indicated NC+59 is the most SO_2-sensitive cultivar. It is concluded that ethylene production is an indicator of SO_2 stress at fumigation levels lower than those that cause visible injury. It is further concluded that differences in ethylene production between cultivars reflect different cultivar sensitivities to SO_2.

6. References

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Table V.1 Ethane formation from broken, intact, and freeze-treated chloroplasts. Standard reaction conditions were used and incubation time was 1 hr in the light.

Chloroplasts	Ethane Production	
	pmol	%
Broken		
$+SO_3^{2-}$	491	100
$+ SO_4^{2-}$	20	4
Intact		
$+SO_3^{2-}$	46	9
$+SO_4^{2-}$	3	1
Freeze-treated		
$+SO_3^{2-}$	2,995	610
$+SO_4^{2-}$	65	13

Table V.2 Effect of DCMU, phenazine methosulfate and methyl viologen on ethane formation and sulfite oxidation from broken chloroplasts. Incubation time for ethane formation was 1 hr in the light. The rate of O_2 uptake was determined from the linear portion of the uptake curve and the small dark rate was subtracted from the light rate.

Addition	Ethane Production	O_2 Uptake
	%	
Sulfite	100[a]	100[b]
Sulfite + DCMU, 10 μM	17	12
Sulfite + PMS, 20 μM	20	18
Sulfite + MV, 100 μM	685	
Sulfate + MV, 100 μM	8	

[a]Ethane production was 284 pmol.

[b]Rate of O_2 uptake was 195 nmol min^{-1}.

Table V.3 Effect of various compounds on ethane formation and sulfite oxidation in broken chloroplasts. Reaction conditions as for Table V.2.

Addition	Ethane Production	O_2 Uptake
	%	
None	100[a]	100[b]
Tiron, 1 mM	3	7
Ascorbate, 3 mM	2	5
Ethanol, 3%	83	
Ethanol, 1%	100	
Formate, 10 mM	133	126
DABCO, 10 mM	110	102
Azide, 10 mM	160	205
Cyanide, 1 mM	135	180

[a]Ethane production was 460 pmol.

[b]Rate of O_2 uptake was 240 nmol min^{-1}.

Table V.4 Effect of superoxide dismutase, catalase and BSA on ethane formation in broken chloroplasts. Incubation time was 1 hr in the light. Chloroplasts containing 300 μg Chl/ml were used.

Addition	Ethane Production
	%
None	100[a]
Superoxide dismutase (30 μg; 60 units)	49
Catalase (30 μg; 1,100 units)	77
BSA (30 μg)	107

[a]Ethane production was 518 pmol.

Table V.5 Ethylene production rates of corn leaves after plants were exposed to air or 0.5 μℓ $ℓ^{-1}$ SO_2 for 6 hr. Leaves were cut from plants immediately (0 hr), 2 hr and 5 hr following the fumigation and ethylene production rates determined over a 1 hr period.

Cultivar	Treatment	Ethylene production rate (nℓ · g^{-1} · hr^{-1}) 0	2	5
Experiment 1				
Bonanza	Air	0	0	0.1
	SO_2	0	0	0
NK 51036	Air	0.1	0	0.1
	SO_2	0	0	0.1
NC+59	Air	0	0.1	0.1
	SO_2	0	1.6	0.2
Experiment 2				
Bonanza	Air	0.2	0.1	0.1
	SO_2	0.1	0.2	0.1
NK 51036	Air	0	0.1	0.2
	SO_2	0.1	0	0.2
NC+59	Air	0.3	0.2	0.1
	SO_2	0.9	0.2	0.2

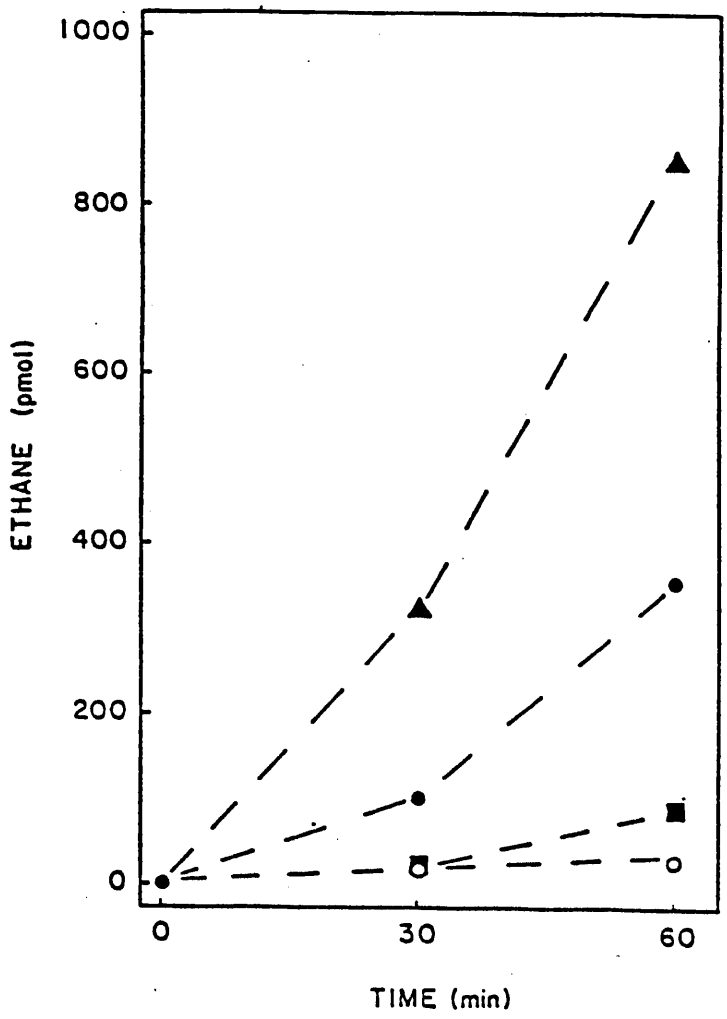

Figure V.1 Ethane production from broken chloroplasts incubated in the light with sulfite (●), sulfite + $FeCl_3$ (100 μM) (▲), sulfite + EDTA (1 mM) (■), or sulfate (○). In the dark, ethane production was less than 20 pmol h^{-1} in all treatments.

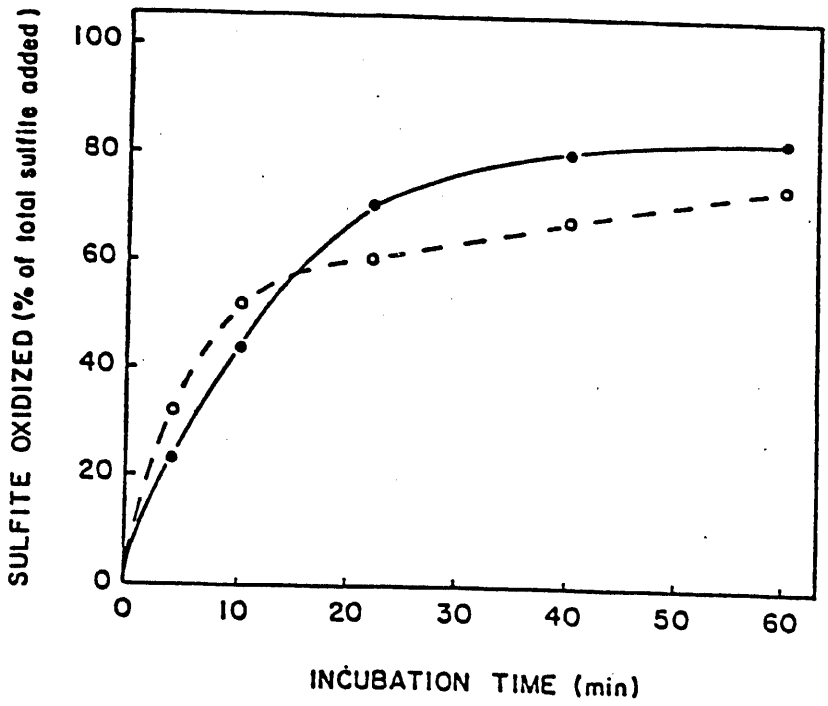

Figure V.2 Sulfite oxidation measured as sulfite loss by the pararosaniline assay during incubation in the light (200 μE m^{-1}) with sulfite (●), or sulfite plus MV (100 μM) (○). In the dark, ethane production was less than 20 pmol h^{-1} in all treatments.

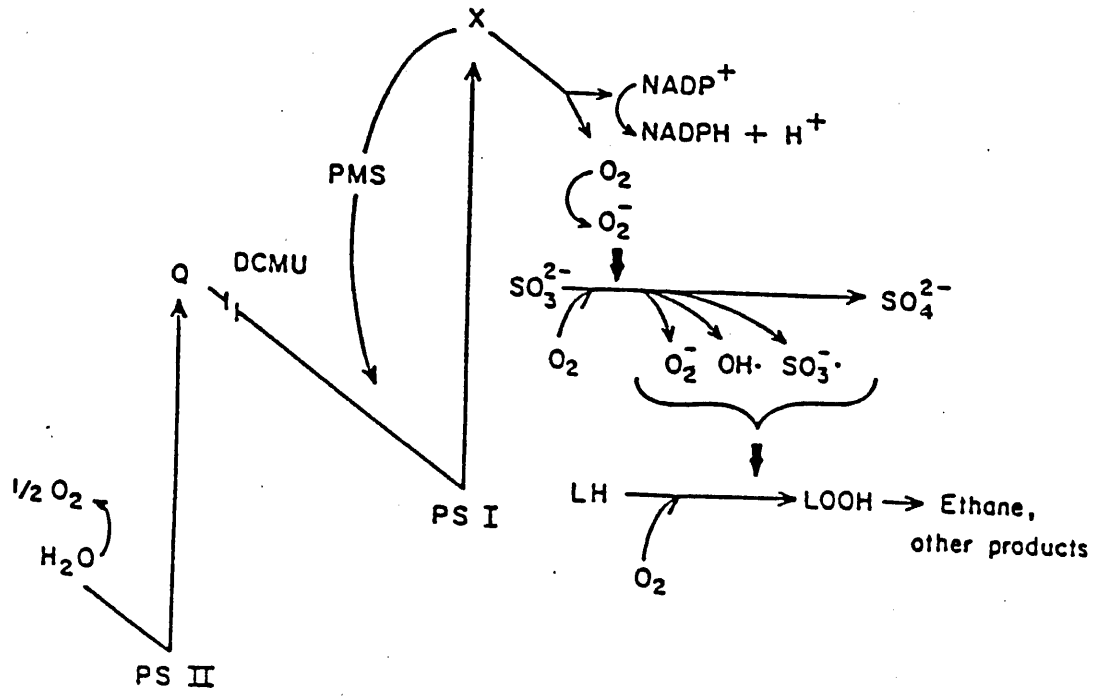

Figure V.3 Proposed scheme for sulfite-induced ethane formation in illuminated chloroplasts. (➡), an initiation reaction.

CHAPTER VI: Snap Bean (Phaseolus vulgaris L.) Plant Growth and Yield Response to Low Level Ozone Exposure

1. Introduction

The reported findings of plant foliar damage and/or plant growth responses to O_3 are based largely upon exposure of plants to moderate and high levels of O_3 (0.1 ppm or higher) (Foster et al., 1983; Jacobson, 1982; Khatamian et al., 1973). Plants were grown in soil or in artificial soil mixtures held in varying size containers in which the soil moisture content and nutrient availability were difficult to maintain uniformly (Armiger et al., 1958). Furthermore the container restricted the root system and inadvertently placed the plant under unknown stress (Timm et al., 1967).

Most findings of plant responses to fumigation of O_3 in a greenhouse and in an outdoor chamber environment were obtained with plants being exposed to O_3 for varying time periods while growing in container held soil. Very few studies subjected plants to O_3 fumigation throughout their life cycle. Direct plant yield comparison between chamber grown plants and field grown plants apparently is very limited. Thus, plant responses attributed to O_3 when plants are grown in containers are suspect (Lewes and Brennan, 1977) and may not be similar to those found in plants under field growing conditions. Growth and productivity of plants grown in field soil and exposed to chronic low levels of O_3 (below 0.1 ppm) throughout their life cycle need to be assessed.

Davis is located between two agriculturally rich valleys, Sacramento and San Joaquin, that yield a major portion of the state's commercial seed of vegetable and agronomic crops, as well as production of vegetable and field crops for fresh market, processing, and animal feed. The ambient air content of O_3 at Davis is low (0 to 0.035 ppm, September 1982). An opportunity existed to measure plant productivity differences that occur under conditions of chronic low level O_3 between field and chamber environments and between optimum water and water-limiting soil environments.

The yield of fruit and seed is reduced when plants are stressed for moisture, yet plants under stress are less sensitive to O_3. The

relative magnitude of change in plant productivity due to moisture stress and O_3 damage (not necessarily visual) needs to be examined more closely. Differences in yield response as well as quality can be determined between plants grown on field soil with and without a chamber environment, and between plants in chambers with filtered air and air containing levels of O_3 below 0.1 $\mu\ell\ \ell^{-1}$. The impact that each plant stress condition contributes to the overall economic value of the crop by yield and quality differences could be determined.

2. Materials and Methods

Four NCLAN-type open-topped fumigation chambers were erected in the field and each was anchored at the base at four opposite points to weights buried in the soil. This was necessary to prevent toppling of chambers during windy periods.

The Yolo sandy loam soil within chamber confine was wetted to a depth of a meter or more by flooding. A week later 35 kg N/ha, as $(NH_4)_2SO_4$, was broadcast and the soil hand spaded to a depth of about 20 cm and leveled. Snap beans, cv. Blue Lakes Stringless 290, reported to be sensitive to ozone (Butler and Tibbitts, 1979), were hand planted June 13 in rows 15 cm apart, positioned north-south, eight rows per chamber. Clear plastic film covering was placed over the upper chamber frame only. The lower half of the frame was covered after plants were thinned to 10 cm apart in the row at first true leaf development. Plant spacing was wider than recommended (Sims et al., 1977) or used in field studies (Bonanno and Mack, 1983) to allow large leaf area development of plants and reduce conditions for fungal (mildew) infection of lower leaves. Sensoring devices to monitor chamber environment along with a drip irrigation system that delivered 2ℓ/hr of water under 20 psi were installed. After the plastic film was securely tied and soil covered at the base, installed blowers were turned on (fuller details of chamber environment monitoring are given in Chapter IA).

Plants were grown at two moisture regimes in each chamber. Tensiometers were positioned at a depth of 15 cm and soil moisture tension recorded daily. Four rows of soil were maintained at -0.05 MPa and the other four rows at -0.1 MPa mean soil moisture tension

(MSMT). At each moisture regime the outer two rows were designated guard rows and the inner two rows were used for plant growth and physiological measurements. Row arrangement and positioning of instrument sensors in a chamber are shown in Fig. VI.1A. Chamber arrangement in the field is also shown, Fig.VI.1B. A similar planting system and instrument positioning was made in two nonchamber sites adjacent to chambers. Chambers were placed south of a long shed that provided electrical outlets and partial protection from the occasional strong north winds yet they would be exposed to full sunlight throughout daylight hours.

Plants were harvested by cutting stems at soil level at three stages of growth, pre-bloom (July 18), bloom (July 31), and at mature fresh bean pods (August 21). The fresh and dry weight, and leaf area of each of five plants were determined at each harvest from each MSMT of all sites. Ten plants were included for yield measurement of fresh bean pods that were graded as mature and immature then counted and weighed.

Plant tissue after weighing was washed in 0.1 N HCl, rinsed twice in distilled water, then dried at 60 C for 72 hours in a forced draft oven. Plant foliage and green pods were prepared separately. After weighing dry tissue was ground in a Wiley mill to pass a 20 mesh screen and stored for chemical analysis. Tissue was analyzed for total calcium and potassium by atomic absorption spectrometer.

A split-split plot design was used to statistically analyze plant growth and green pod yield, and plant nutrient content. The physiological responses of plants measured during growth are reported in Chapter VII.

3. Results

Plant Growth and Bean Pod Yield

As shown in Table VI.1 growth of bean plants by bloom (July 31) in chambers without O_3 fumigation was comparable to field grown plants. At fresh green pod harvest (August 21), however, field grown plants displayed greater fresh weight and leaf area than chamber grown plants. Plants in chamber environs tended to be somewhat larger with smooth, fully extended leaf edges whereas field plants were somewhat

shorter with leaves that had crinkled edges and rougher surface. Fresh green pod yield was not significantly different between chamber and field grown plants (Table VI.2).

For the first 20 days (prebloom) there was no discernible difference in development of plants in chambers with or without ozone fumigation. By 33 days (bloom) plants exposed to ozone showed some sign of growth alteration by a decline in enlargement with a gradual overall leaf color change from deep green to a lighter green intensity. At fresh green pod harvest (August 21) 54 days of ozone fumigation had an adverse effect on plant growth as evident by lower plant fresh weight yield, lesser leaf area, and a lower fresh pod number and yield (Table VI.2) than found with plants grown in chambers without ozone fumigation.

Plant Nutrition

Plant uptake of calcium and potassium was not significantly different among plants grown in chamber and field environment. As shown in Table VI.5 plant content of calcium increased and that of potassium decreased during growth. At final harvest calcium was between 3 to 4 times higher than potassium. However, the potassium content in green pods was about twofold greater than calcium. Furthermore potassium in plants and green pods was nearly similar whereas calcium in plants was between 7 to 9 times higher than in green pods. In one instance only, at final harvest, was a lower calcium content found in plants grown in field environment than in chamber environment.

4. Discussion

Our findings indicate that a marked decline in plant growth and lower total fresh green pod yield of snap bean cv. Blue Lakes Stringless 290 was due primarily to daily 5-hour fumigation with 0.07 to 0.10 $\mu\ell\ \ell^{-1}$ ozone for 54 days (Tables VI.1 and VI.2). A lesser magnitude of loss in plant productivity was attributable to chamber environment (Tables VI.1 and VI.2) and soil moisture status (Table VI.3).

Effect of Ozone

Ozone fumigated plants developed 25% smaller leaf area and produced about 41% lower total fresh green pod and mature green pod

weight and 30% fewer mature green pods than nonfumigated plants. No significant differences were revealed among green pod yield components between chamber and nonchamber field grown plants. Yet differences in plant growth became evident with time. By bloom (July 31) plant growth in nonfumigated chambers was nearly similar to field grown plants. However, between bloom and harvest of green pods (August 21) 21 days of growth was more favorable for field than chamber grown plants. This was evident by the increase in plant fresh weight and larger leaf area (Table VI.1). These responses were found despite the low number of plants, 5 per plant growth measurement and 10 per fresh green pod yield determination, that were available. Plant numbers were limited because each chamber contained two soil moisture regimes from which plants were utilized for not only plant productivity measurements but also for physiological determinations. Physiological changes in plants attributable to O_3 injury (Craker and Starbuck, 1972; Perchorowicz and Ting, 1974; Butler and Tibbitts, 1979) to account for the observed decline in plant growth, were not clearly revealed. Considerable variability existed among the few plants available for testing and only a trend was discernible (Chapter VII). The change in foliar color, from a dark green to a yellowish green, observed during plant growth was likely due to O_3 injury effecting a gradual degradation in chlorophyll synthesis (Knudson et al., 1977; Olszyk and Tibbitts, 1982) that resulted in a slow decline in the level of carbohydrate production (Jacobson, 1982). The reduction in plant leaf area with time (Table VI.1) without the development of necrotic lesions, and lower green pod yield (Table VI.2) are symptoms of exposure to no greater than 0.1 $\mu\ell\ \ell^{-1}$ O_3.

Plants expend energy for nutrient absorption and for root expansion (Manning et al., 1971; Jacobson, 1982). The probable reduction in carbohydrate production was not limiting plant uptake of calcium or potassium since no differences in plant content of either element was found among plants grown in chamber or field environment (Table VI.5). Therefore plant growth was not limited by inadequate nutrition (Mack, 1983). The Yolo sandy loam soil in which plants were grown is inherently fertile (Zahara and Timm, 1973) and had received a recommended level of fertilizer (Sims et al., 1977).

Effect of soil moisture

Snap beans are classified as having shallow rooting characteristics. The greater portion of their rooting occurs in the upper 15 to 30 cm of the soil. Tensiometers positioned at 15 cm reflect the changes in soil moisture removed by plant roots in a manner similar to that of another shallow rooted crop, potatoes (Flocker and Timm, 1966). Snap bean production can be modified by the status of available soil moisture (Drake and Silbernagel, 1982 and Mack and Varseveld, 1982). Weaver et al. (1984) demonstrated on a Yolo sandy loam soil that bean yield of several cultivars was markedly reduced when the MSMT was reduced from -0.05 MPa to -0.1 MPa, the range of soil moisture tensions used in this study. Fresh green pod yield was significantly reduced in plants grown at -0.1 MPa compared to -0.05 MPa (Table VI.3) yet plant growth was unaffected. Temporary moisture stressed plants can be adversely affected by reduced flower set and/or pod formation (Weaver et al., 1984, Karlen et al., 1982, Stansell and Smittle, 1980) which accounts for in part, but not totally, the lower yield of green pods.

Effect of ozone and soil moisture combined

An interactive effect was found between O_3 fumigation and MSMT on total pod fresh weight yield. Plants grown at -0.05 MPa MSMT out-yielded plants grown at -0.1 MPa MSMT in chambers without O_3 fumigation and field grown (Table VI.4). Total green pod yield of plants in O_3 fumigated chambers was similar at -0.05 and -0.1 MPa MSMT. The lower yield due to soil moisture regime (Table VI.3) was compounded by the effect of O_3 fumigation with a greater yield decline at -0.05 MPa than at -0.1 MPa MSMT. The decline in yield was about half at -0.05 MPa and about one-third at -0.10 MPa MSMT of plants in O_3 fumigated chambers than found with nonfumigated chambers.

Implication of short- and long-term ozone exposure on plant productivity

The snap bean cultivar used in this study had determinate characteristics; little or no increase in foliar growth after pod set. Plant growth was relatively short, 68 days from planting of seed to harvest of green pods. Yet plant growth and green pod yield were significantly affected by low level fumigation of ozone for 54 days

(Tables VI.1 and VI.2), and by soil moisture conditions (Table VI.3). The effect of chamber versus field environment was not as clearly significant early in plant growth but was significant at green pod harvest (Tables VI.1 and VI.4). It would seem more than likely that, had foliar growth continued, along with flower formation and pod development that is characteristic of indeterminate plant type, the effect of ozone on plant productivity would have been more severe. Stressed plants yield lower numbers of seed and weight of seed (Weaver et al., 1984). The magnitude of seed yield loss of plants to low level ozone exposure is still questionable (Lewis and Brennan, 1977).

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Table VI.1. Bean plant growth response to chamber environment of filtered air without and with ozone fumigation, and non-chamber field environment.[1]

Plant growth environment	Harvest date	Plant[2]			Leaf[2]		
		Length (cm)	Fresh wt (g)	Dry wt (g)	Number	Area (cm^2)	Area / Number
In chamber-filtered air minus O_3	July 18 (pre-bloom)	26.5a	49.1a	6.0a	34.3a	1258.2a	36.0a
In chamber-filtered air plus O_3		22.1a	37.4a	4.8a	30.9a	968.8a	31.9a
Non-chamber field		19.7a	40.3a	5.5a	34.5a	1003.1a	29.0a
In chamber-filtered air minus O_3	July 31 (bloom)	38.6a	127.7a	16.6a	57.7a	2654.7a	48.2a
In chamber-filtered air plus O_3		30.9b	78.5a	11.9a	47.8a	1685.8a	35.3a
Non-chamber field		32.7a	112.1a	14.4a	59.0a	2233.7a	37.9a
In chamber-filtered air minus O_3	August 21 (mature green pods)	--	112.5b	39.9a	46.1a	2300.7b	51.5a
In chamber-filtered air plus O_3		--	82.1b	35.8a	43.6a	1732.3c	39.8a
Non-chamber field		--	146.7a	46.0a	59.2a	2900.0a	49.4a

[1]Fumigation with ozone at 0.07 to 0.10 $\mu\ell\ \ell^{-1}$ for 5 hours daily.

[2]Means followed by a common letter are not significantly different at the 5% level within harvest date. Based on mean of 5 plants. Green bean pod yield not included in August 21 harvest.

Table VI.2. Green bean pod yield response to chamber environment of filtered air without and with ozone fumigation, and non-chamber field environment.[1,2]

Plant growth environment	Total pod wt (g)	Mature green pod wt (g)	Number of Pods	
			Immature	Mature
In chamber-filtered air minus O_3	125.8a	118.7a	9.9b	22.1a
In chamber-filtered air plus O_3	74.7b	69.6b	7.5c	15.0b
Non-chamber field	130.7a	119.4a	13.7a	20.7a

[1]Fumigation with ozone at 0.07 to 0.10 $\mu\ell\ \ell^{-1}$ for 5 hours daily for 54 days.

[2]Means followed by a common letter are not significantly different at the 5% level. Based on mean of 10 plants.

Table VI.3. Effect of mean soil moisture tension on bean plant growth and fresh green pod yield.[1]

Soil moisture tension[2]	Plant wt (g)		Leaf		Pod fresh wt (g)		Pod number	
	Fresh	Dry	Number	Area (cm^2)	Total	Mature	Immature	Mature
-0.05 MPa	112.9a	40.1a	49.3a	2313.2a	122.2a	113.4a	10.2a	20.4a
-0.1 MPa	114.6a	41.1a	49.9a	2308.9a	98.6b	91.8b	10.5a	18.1a

[1]Means followed by a common letter are not significantly different at the 5% level. Pooled data from chamber and non-chamber grown plants.

[2]Tensions recorded at 15 cm soil depth.

Table VI.4. Interactive effect of chamber environment of filtered air without and with ozone fumigation and non-chamber field environment, and soil moisture tension on total green bean pod yield.[1]

Plant growth environment	Soil moisture tension[2] (MPa)	Total pod fresh wt./plant[3] (g)
In chamber-filtered air minus O_3	-0.05	132.7a
	-0.1	118.8b
In chamber-filtered air plus O_3	-0.05	74.7c
	-0.1	74.7c
Non-chamber field	-0.05	159.1a
	-0.1	108.2b

[1]Fumigation with ozone at 0.07 to 0.10 $\mu\ell\ \ell^{-1}$ for 5 hours daily for 54 days.

[2]Tensions recorded at 15 cm soil depth.

[3]Means followed by a common letter are not significantly different at the 5% level.

Table VI.5. The calcium and potassium content of bean plants during growth and in green bean pods at harvest in chamber environment without and with ozone fumigation, and non-chamber field environment.[1,2]

Plant growth environment	Percent calcium				Percent potassium			
	Plant			Green pod	Plant			Green pod
	July 18	July 31	August 21	August 21	July 18	July 31	August 21	August 21
In chamber-filtered air minus O_3	1.45a	2.56a	4.73a	0.39a	3.47a	1.47a	0.95a	0.87a
In chamber-filtered air plus O_3	2.19a	2.64a	4.61a	0.51a	2.83a	1.40a	1.03a	0.89a
Non-chamber field	1.69a	2.46a	2.93b	0.41a	3.21a	1.49a	0.87a	0.93a

[1]Fumigation with ozone at 0.07 to 0.10 $\mu\ell\ \ell^{-1}$ for 5 hours daily.

[2]Means followed by a common letter are not significantly different at the 5% level within dates.

Table VI.6. Effect of mean soil moisture tension on bean plant and fresh green pod content of calcium and potassium.[1]

Soil moisture tension (MPa)[2]	Nutrient content (%) mg/100g D.W.			
	Plant		Green pod	
	Ca	K	Ca	K
-0.05	4.05a	1.04a	0.45a	0.92a
-0.1	4.13a	0.86b	0.42a	0.87a

[1]Means followed by a common letter are not significantly different at the 5% level. Pooled data from chamber and non-chamber grown plants.

[2]Tensions recorded at 15 cm soil depth.

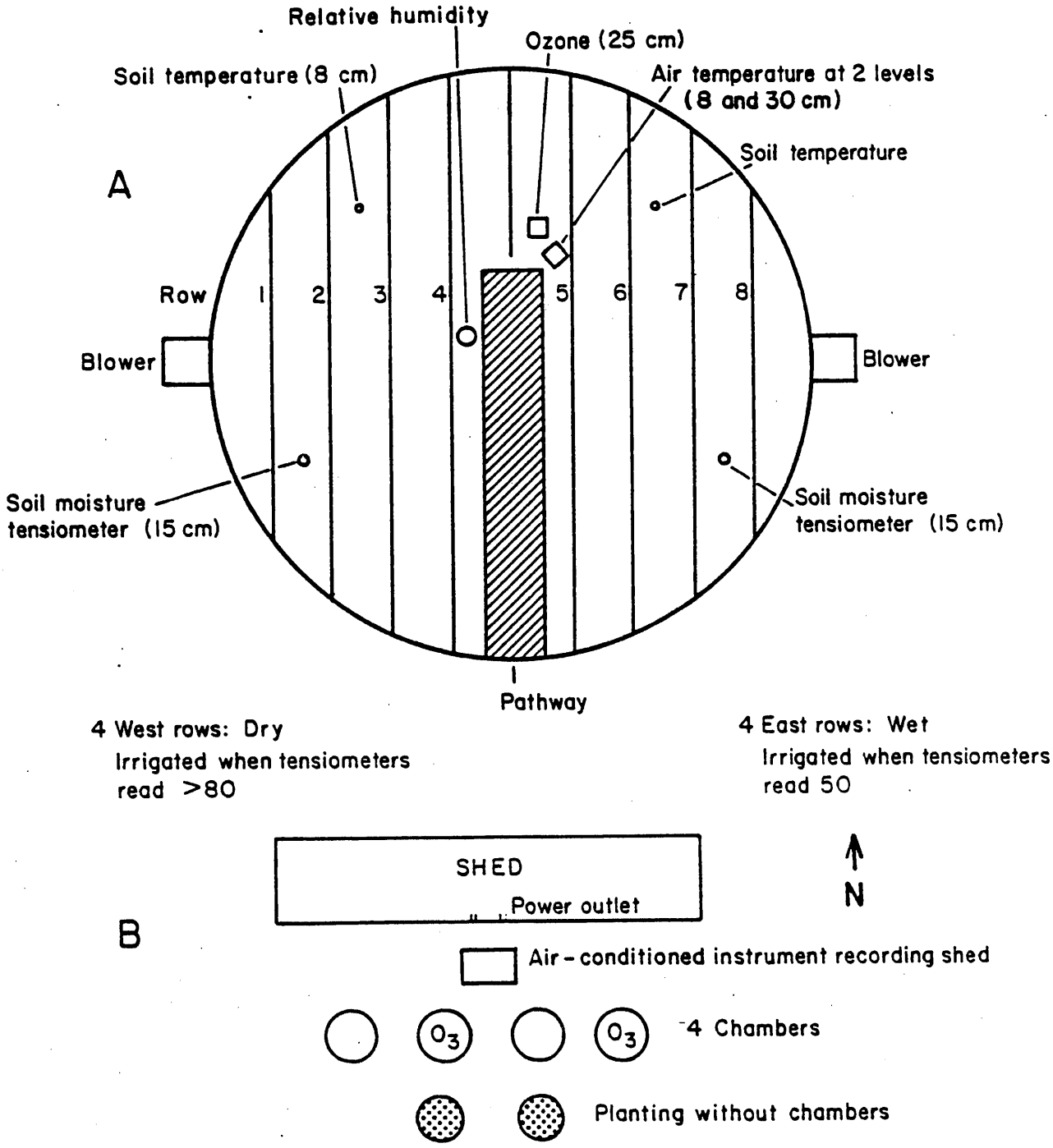

Figure VI.1. Schematics of experimental arrangement of A - Plant row numbers and placement of sensors to measure environment within chamber and in non-chamber field site, and B - Position of chambers and non-chamber planted sites partially protected by a large shed against gusty north winds. Electrical power was supplied to a small shed housing instruments that recorded data from sensors, and equipment for ozone fumigation and for continuous monitoring of ozone level during plant growth.

CHAPTER VII. Growth Chamber and Field Chamber-- Physiological Processes

1. Introduction

The physiological responses of bean plants (Phaseolus vulgaris L.) to air pollution were investigated in two phases: a) differences among cultivars that had been previously described as differentially sensitive to visible injury (Butler and Tibbitts, 1979a), and b) a field study with one cultivar grown under different water regimes.

The uptake of a given air pollutant by a plant is determined primarily by the stomatal aperture. Therefore, it would follow that the water status of the plant during fumigation would have a pronounced effect on the amount of the pollutant absorbed by the plant and subsequent injury. Plants subjected to water stress have been shown to exhibit less injury when exposed to air pollution than plants grown under optimal moisture conditions (Olszyk and Tibbitts, 1981b; Tingey et al., 1982). Both investigations concluded that decreased stomatal conductance provided protection from injury. Ozone sensitivity was attributed to differences in stomatal mechanisms of resistant and sensitive bean cultivars grown under identical conditions in a growth chamber (Butler and Tibbitts, 1979b). In contrast to the aforementioned investigations, Evans and Ting (1974) concluded that internal characteristics determined the most sensitive age of a bean leaf to ozone, not stomatal mechanisms. Olszyk and Tibbitts (1981a) investigated the relationship between stomatal aperture and visible injury in pea plants exposed to ozone and sulfur dioxide, alone or in combination, and concluded that the changes in stomatal aperture were not correlated with the degree of leaf injury produced at different pollutant levels. Another investigation using bean plants concluded that stomatal responses did not explain the difference in injury development between O_3 alone and O_3 and SO_2 combined (Beckerson and Hofstra, 1979). These seemingly conflicting conclusions may be the result of various factors, such as species differences, growth conditions, type of pollutant, exposure concentration and duration, and differences in environmental conditions during air pollutant exposure.

Disruption of membrane function has been suggested as a primary effect of ozone on plant cells (Perchorowicz and Ting, 1974; Heath, 1975). In soybean and white bean leaf strips exposure to O_3 resulted in increased electrolyte leakage compared to controls, while the combination of SO_2 and O_3 did not increase leakage above that of the controls (Beckerson and Hofstra, 1980). The leakage of potassium, calcium, sugars, and amino acids from leaf discs of a sensitive bean cultivar was much greater than from a tolerant one 24 hours after exposure to ozone (McKersie, Hucl, and Beversdorf, 1982). It is not clear if the difference in leakage may have been due to differential uptake of ozone by the two cultivars. Sulfur dioxide has been shown to cause increased K^+ efflux from lichens, but this was at very high levels of aqueous SO_2 (Puckett et al., 1977).

A plant's ability to cope with air pollution stress involves avoidance by stomatal mechanisms and tolerance at the cellular level. We have tried to characterize various physiological processes in differentially sensitive bean cultivars and in bean plants grown under different water regimes and exposed to long-term, low-level ozone treatments.

2. Materials and Methods

a) Growth Chamber

Four cultivars of bean (Phaseolus vulgaris) were used in our experiments: Bush Blue Lake 290 (BBL), Black Turtle Soup (BTS), French's Horticultural (FH) and Spurt (S). BBL and S are ozone-sensitive and BTS and FH are ozone-resistant bean cultivars, respectively. The seeds were imbibed for 24 hours in deionized, distilled water in the dark at room temperature. Then, they were sandwiched between two layers of absorbent paper, moistened with 0.5 mM $CaSO_4$ and the paper containing the seeds made into rolls. The seed rolls were then placed in covered containers in the dark at room temperature for three days. On the third day, the roots were re-moistened with 0.1 strength modified Hoagland (Epstein, 1972) solution with FE-EDTA at 0.2 strength. Seven days from imbibition, the seeds were transferred to 1 gallon containers filled with 0.5 strength modified Hoagland solution with Fe-EDTA at full strength.

The solutions were aerated with filtered air. Four plants were transferred per container. The plants were grown for 4 days in a growth chamber with the environmental conditions set to match the conditions of the fumigation chambers, then transferred to the fumigation chambers (described in Chapter III).

Environmental conditions in the fumigation chambers were as follows: quantum flux density 250 ± 10 μ Einsteins $m^{-2}s^{-1}$; 25.8 ± 1°C day/20.5 ± 0.5°C night temperature; 65 ± 5% relative humidity day and night; and a 16 hour photoperiod. Air flow through the fumigation chamber was set at one volume exchange per minute. Fumigations were routinely initiated 4 hours into the photoperiod.

Diffusive conductance of stomates was measured with a Lambda autoporometer and LI-20s horizontal sensor. Calibration of the sensor was performed according to T. C. Hsiao (personal communication). The sensor was stored in a desiccator over silica gel between experiments. Prior to measurements, the sensor was placed into the exposure chambers and allowed to come to thermal equilibrium. Measurements were made through glove ports in the sides of the exposure chambers.

Ion leakage was determined by measuring the change in electrical conductivity of incubation solutions containing discs from SO_2 and/or O_3 fumigated leaves and non-fumigated leaves. Ion leakage is taken as a measure of air pollutant-induced leakage of leaf cell membranes. Approximately 20 leaf discs were punched from each of the two primary leaves of each bean plant with a No. 2 cork borer (diameter = 5.6 mm) immediately or 24 hours after fumigation. The leaf discs were washed in a 0.2 mM $CaSO_4$ solution for one hour then transferred to 20 ml plastic vials containing 10 mls of either deionized, distilled water or 0.2 mM $CaSO_4$; calcium has been shown to be important for membrane integrity (Läuchli and Epstein, 1971). Twenty leaf discs were placed in each vial. The electrical conductivity change of the bathing solution was determined with a YSI model 32, electrical conductivity meter provided with a 0.1 cm, pipette type, conductivity cell. All measurements were made with the solutions at 25°C. At the end of the four hour incubation period the leaf discs were frozen and thawed and their electrical conductivity measured to determine the total conductivity of the tissue.

Percent leakage shown in Figures 3-17 and 22-24 was calculated from the following formula:

$$\frac{v_{tx} - v_{to}}{v_{tf} - v_{to}} \times 100 = \% \text{ leakage}$$

where v_{tx} = conductivity at various times after the initial incubation of leaf discs.

v_{to} = conductivity of solutions before discs were added.

v_{tf} = conductivity after leaf discs were frozen and returned to 25°C.

Fumigation treatments for examining stomatal conductance were for 2.5 hours with 0.1 $\mu\ell\ \ell^{-1}$ O_3 or 0.4 $\mu\ell\ \ell^{-1}$ SO_2. Ion leakage was analyzed from plants exposed to 0.1 $\mu\ell\ \ell^{-1}$ O_3 and 0.4 $\mu\ell\ \ell^{-1}$ SO_2 singly or in combination for three hours.

Data were statistically tested by analysis of variance.

b) Field Study

Field chambers and outside plots were prepared as specified in Chapter VI. Beans, cv. Bush Blue Lake (ozone-sensitive cultivar), were sown June 13, 1984. Two soil water regimes for each plot were imposed through the use of a drip irrigation system equipped with shut-off valves. The side walls of the chambers were removed at sowing to prevent heat stress effects on young seedlings. On June 27, the chambers were enclosed and fumigation commenced two days later. Ozone treatments were 0.07 to 0.1 $\mu\ell\ \ell^{-1}$ O_3 for five hours daily. Ozone was generated by a silent arc ozonator (Model 03V10-0; Ozone Research and Equipment Corp., Phoenix, AZ) and analyzed by a Dasibi Ozone Monitor (Models 1003AH and 1003PC). Ozone was sampled through Teflon tubing (0.25 in. o.d.) at the center of each chamber and at two points adjacent to the field chambers.

Physiological and growth (Chapter VI) parameters were analyzed at three growth stages: pre-bloom, bloom, and mature pods. Physiological parameters investigated were stomatal conductance, leaf temperature, leaf water potential, and ion leakage from leaf discs.

Stomatal Conductance.

Measurements were taken during the fumigation period with a steady-state porometer (Model LI-1600C; Li-Cor, Inc., Lincoln, NE).

The abaxial side of expanded leaves was sampled for stomatal conductance and leaf temperature. Leaf temperature was measured concurrently with a thermocouple in the porometer cuvette in contact with the leaf surface. Eight to twelve samples were taken per plot. The same leaves were subsequently used for ion leakage and leaf water potential measurements.

Leaf Water Potential.

Water potential was measured with the standard pressure bomb. Trifoliate leaves were excised with a razor blade and placed in plastic bags. They were immediately transferred to the pressure bomb chamber. A moist paper towel was placed in the chamber to prevent transpiration from the leaves. Three to five samples were taken from each plot.

Leaf Temperature.

Leaf temperature was measured by two techniques. The first was described under stomatal conductance. This involved a thermocouple in contact with the abaxial surface of the leaf. Eight to twelve samples were recorded for each plot. The second technique utilized an infrared thermometer (Model 210, Everest Interscience, Tustin, CA). These readings tended to be variable due to factors of leaf orientation and canopy characteristics.

Ion Leakage.

An expanded trifoliate leaf was excised and placed in a plastic bag. Twenty leaf discs were then cut with a No. 2 cork borer. Leaf discs were placed in a cold 0.2 mM $CaSO_4$ incubation solution for one hour. The discs were then rinsed with distilled, deionized water and placed in 10 ml of a 0.2 mM $CaSO_4$ solution at 25°C in a shaking water bath. The electrical conductivity of this solution was immediately measured with a pipette-type conductivity cell (cell constant = 0.1 cm) and a YSI Conductivity Meter (Model 32; Yellow Springs Instrument Co., Yellow Springs, OH). Between samples the cell was thoroughly rinsed with distilled, deionized water. Readings were taken hourly for four hours. At four hours, 1 ml of the solution from each sample was withdrawn and frozen for K analysis. One ml of fresh 0.2 mM $CaSO_4$ was then added to the original samples and the samples subsequently frozen. The next day this solution was thawed and

brought to 25°C. Total electrical conductivity of this solution was then measured. Calculations took the 10% dilution into account.

Percent leakage was calculated as for the growth chamber study, with the exception that v_{tf} = conductivity after leaf discs were frozen and returned to 25°C, plus 10% correction.

Potassium analysis was carried out in a solution containing La and Cs by flame emission spectrophotometry.

Data were statistically tested by analysis of variance.

3. Results

a) Growth Chamber

Low level ozone exposure (0.1 $\mu\ell\ \ell^{-1}$, 2.5 hr.) caused significant increases in stomatal conductance in the two sensitive bean cultivars (BBL and S) but this ozone induced stomatal opening did not cause immediate visible injury. There was no significant stomatal response due to ozone exposure in the two resistant cultivars (Table VII.1).

Stomatal conductances were measured at various times before and after exposing bean plants to 0.4 $\mu\ell\ \ell^{-1}$ SO_2 (Figs. VII.1a and b). Although there was no visible injury in any of the cultivars, in the BBL cultivar (Fig. VII.1a), the SO_2 initially reduced stomatal conductance but later appeared to have no effect. Stomatal conductance in the SO_2 treated BTS cultivar, in contrast, was not significantly reduced until after 2.5 hours of SO_2 fumigation (Fig. VII.1b). In the FH plants, stomatal conductance was enhanced after a half-hour exposure to SO_2 then reduced to the level of the controls with further exposure to SO_2 (Fig. VII.2a). Increased stomatal conductance over the control level was also observed in the S cultivar, however, this rise in conductance was not apparent until the conclusion of the fumigation period (Fig. VII.2b).

Ion leakage from leaf discs prepared immediately after exposure to 0.1 $\mu\ell\ \ell^{-1}$ O_3 for three hours exhibited a significantly higher than control rate of ion leakage from BBL leaf discs regardless of whether the leaf discs were incubated in deionized, distilled water or 0.2 mM $CaSO_4$ (Figs. VII.3a,b). In the BTS cultivar, leakage was suppressed by the O_3 treatment for both distilled deionized and 0.2 mM $CaSO_4$ incubated leaf discs (Figs. VII.4a,b). In the FH cultivar a

significant increase in leakage was only observed when the leaf discs were incubated in distilled, deionized water but not when incubated in 0.2 mM $CaSO_4$ (Figs. VII.5a,b). The leaf discs of the S cultivar displayed no significant difference in conductivity when exposed to O_3 (Figs. VII.6a,b).

A significant increase in ion leakage was measured from BBL leaf discs immediately after exposure to 0.4 $\mu\ell\ \ell^{-1}$ SO_2 for three hours. Ion leakage into $CaSO_4$ was much less than into deionized water, but was also increased by SO_2 (Figs. VII.7a,b). BTS leaf discs given the same SO_2 treatment also displayed increased ion leakage when incubated in deionized distilled water but not when incubated in 0.2 mM $CaSO_4$ (Figs. VII.8a,b). In two separate experiments on the effects of SO_2 on ion leakage from FH plants, variable results were obtained (not shown). Leakage from S cultivar discs treated with 0.4 $\mu\ell\ \ell^{-1}$ SO_2 for three hours was significantly suppressed when the discs were incubated in distilled, deionized water but not in 0.2 mM $CaSO_4$ (Figs. VII.9a.b).

Some evidence exists (McKersie, Hucl, and Beversdorf, 1982) suggesting that the effect of ozone on membranes may be secondary and takes some time to develop. Subsequently a series of experiments was carried out in which leaf discs were prepared 24 hours after a single exposure to O_3 and SO_2, singly or in combination. Exposure to 0.1 $\mu\ell\ \ell^{-1}$ O_3 or 0.4 $\mu\ell\ \ell^{-1}$ SO_2 for three hours stimulated ion leakage from leaf discs from both the BBL and BTS cultivars (Figs. VII.10a,b; VII.11a,b: VII.12a,b; and VII.13), but not from the FH or S cultivars (data not shown).

Combined fumigations of 0.1 $\mu\ell\ \ell^{-1}$ O_3 and 0.4 $\mu\ell\ \ell^{-1}$ SO_2 for three hours stimulated ion leakage from all 4 bean cultivars (Figs. VII.14a,b-VII.17a,b). Ion leakage from the BBL cultivar (Fig. VII.14a) was similar to leakage stimulated by O_3 fumigation alone (Fig. VII.10a). However, with the BTS cultivar, the combined SO_2 and O_3 treatment enhanced ion leakage (Figs. VII.15a,b) above the levels of either the O_3 or SO_2 treatments alone (Fig.s VII.12a,b; VII.13). The combined SO_2 and O_3 exposures also stimulated ion leakage from both the FH and S cultivars (Figs. VII.16a,b; VII.17a,b)

although exposures to either of these pollutants alone did not stimulate leakage.

For all cultivars, incubation of the leaf discs in 0.2 mM calcium solutions reduced ion leakage as compared with discs incubated in deionized water (Figs. VII.14a,b-VII.17a,b).

b) Field Study

Stomatal conductance was measured at three stages of development on the abaxial side of bean leaves cv. Bush Blue Lake (O_3-sensitive). Variability within treatments and the length of time involved taking measurements confounded the results to a large degree. At the pre-bloom stage (Fig. VII.18) no significant differences in stomatal conductance were induced by O_3. There was a slight trend toward higher conductances by the well-watered plants of all three treatments. Plants at the flowering stage (Fig. VII.19) followed the same trend as mentioned previously, but again there were no significant differences between control and O_3 treatments. At the harvest stage (Fig. VII.20), plants fumigated with ozone had lower stomatal conductance when well watered, but variability prevented the statistical separation of the treatments. Conductivity of plants grown under ambient conditions appears higher particularly under dry conditions (Figs. VII.18-20), but this may be due to the slower development of these plants.

Leaf water potentials were measured during the afternoon at the pre-bloom growth stage and in the morning for the last two harvests. Leaf water potential at the pre-bloom stage (Fig. VII.21) is characterized by generally more negative water potentials for the plants grown under the dry water regime than the wet one. Ozone-fumigated plants had significantly more negative leaf water potentials than the other two treatments under the dry water regime and than the control chamber grown plants under wet conditions (Fig. VII.21). There was no significant difference between plants grown in filtered air and ambient air under either water regime. No statistically significant differences were apparent among treatments at either the flowering or harvest stages of growth (Fig. VII.21).

The percent ion leakage over a four hour period is illustrated in Figs. VII.22-24. The scale of the y-axis should be noted as leakage

tended to increase with age of the plants. Despite the disparity in percent leakage, statistical analyses failed to separate treatments from one another. Generally, plants grown under non-water stressed conditions had less leakage than those stressed. At the pre-flowering stage there was no difference among the treatments under either water regime (Fig. VII.22). At the flowering stage (Fig. VII.23) plants treated with ozone under both water regimes displayed somewhat higher leakage than plants from the other two treatments. Unexpectedly, ozone fumigated plants had the greatest amount of leakage under water-stressed conditions. Whether this indicates that the degree of water stress these plants were experiencing was harmful in itself and overriding the effects of the ozone is beyond the scope of this study. Ion leakage at the final harvest is illustrated in Fig. VII.24. The low level of leakage in plants grown under ambient conditions may be due to their slightly retarded rate of development, compared to those grown in the field chambers (with leaves undergoing senescence). Ion leakage appears extraordinarily high in plants grown in the field chambers. Plants fumigated with ozone had a somewhat higher leakage than those grown in filtered air (Fig. VII.24).

Potassium leakage was measured from leaf discs after a four hour incubation period. At both pre-bloom and flowering (Figs. VII.25,26) an interaction seems to exist even though it is not apparent in the statistical analyses performed. Potassium leakage was reduced to a greater degree in non-water stressed plants not exposed to ozone than in plants exposed to ozone. This phenomenon was also apparent at the final harvest when comparing the filtered air plants to those exposed to ozone (Fig. VII.27).

4. Discussion

a) Growth Chamber

Stomatal responses of the four bean cultivars to short-term, low-level ozone fumigation are shown in Table VII.1. The two sensitive cultivars (Bush Blue Lake and Spurt) had significantly higher stomatal conductance following fumigation, whereas the two resistant cultivars (Black Turtle Soup and French's Horticultural) were not significantly affected (Table VII.1). Butler and Tibbitts

(1979b) observed stomatal closure in both Black Turtle Soup and French's Horticultural at ozone levels causing visible damage (1.34 $\mu\ell\ \ell^{-1}$ for one hour), but stomatal opening response was observed for the sensitive cultivars (Bush Blue Lake and Spurt). This discrepancy between our results and those of Butler and Tibbitts (1979b) may be due to different fumigation conditions and cultural practices. It is interesting that despite the different results the two sensitive cultivars appeared to be exposed in our study to higher levels of ozone because of higher stomatal conductance. Pea plants exposed to low-level ozone (0.13 $\mu\ell\ \ell^{-1}$) exhibited stomatal opening after an eight hour exposure but not after one to two hours (Olszyk and Tibbits, 1981a). Stomatal closure was observed at higher, injurious levels of ozone for pea plants (Olszyk and Tibbitts, 1981a).

The relative sensitivity of the four bean cultivars to SO_2 had not been studied before. Stomatal responses of the cultivars was followed throughout a 2.5 hour fumigation of SO_2 at 0.4 $\mu\ell\ \ell^{-1}$. There was no apparent visible injury for any of the cultivars. Olszyk and Tibbits (1981a) observed stomatal closure in pea plants exposed to injurious levels of SO_2. Other studies have reported conflicting responses to SO_2 exposure (Kondo and Sugahara, 1978; Noland and Kozlowski, 1979; Rosen, Musselman and Kender, 1978). The four bean cultivars investigated here responded differently to SO_2 exposure (Figs. VII.1a,b and VII.2a,b). The stomatal conductance of cv. Bush Blue Lake was initially depressed by SO_2 exposure but returned to control levels soon thereafter (Fig. VII.1a) while cv. Black Turtle Soup was unaffected until the end of the fumigation period at which time conductance was reduced below that of the control plants (Fig. VII.1b). For the cultivar French's Horticultural stomatal conductance was enhanced after 0.5 hour exposure to SO_2, but thereafter returned to control levels (Fig. VII.2a). Stomatal opening in cv. Spurt was not apparent until the conclusion of the fumigation period (Fig. VII.2b).

In defining the sensitivity of a plant to gaseous air pollutants, not only is the control of absorption important, but the ability of the plant to detoxify the pollutant internally may be of equal importance. Plant cell membranes have been recognized as a primary target

of ozone toxicity. Increased leakiness of membranes has been observed in numerous species of plants exposed to ozone (Perchorowicz and Ting, 1974; Chimiklis and Heath, 1975; and McKersie, Hucl and Beversdorf, 1982). In an investigation utilizing soybean, white bean, cucumber, and radish, SO_2 fumigation did not increase membrane leakiness above that of controls, whereas exposure to ozone increased leakiness from leaf strips of soybean and white bean, but not radish and cucumber (Beckerson and Hofstra, 1979).

Ion leakage, measured as electrical conductivity, from leaf discs of the four bean cultivars was measured during a four hour period in incubation solutions of deionized water or 0.2 mM $CaSO_4$ immediately or 24 hours after fumigation with O_3 and SO_2, singly or in combination. Exposure to 0.1 $\mu\ell\ \ell^{-1}$ O_3 for three hours resulted in significantly increased ion leakage for the sensitive cultivar Bush Blue Lake in both incubation solutions (Fig. VII.3a,b) immediately after fumigation. The other sensitive cultivar, Spurt, displayed no significant increase in conductivity when exposed to O_3 immediately after fumigation (Fig. VII.6a,b). Ion leakage in the resistant cultivar Black Turtle Soup was significantly lower in O_3-treated plants than in control plants immediately after fumigation (Fig. VII.4 a,b). The cultivar French's Horticultural had significantly higher ion leakage in O_3-treated plants when incubated in deionized water but not when incubated in 0.2 mM $CaSO_4$, when measured immediately after fumigation (Fig. VII.5a,b). This suggests a protective effect of Ca^{2+} on membrane integrity in the latter cultivar (see Epstein, 1961).

Ion leakage was increased in plants treated with 0.4 $\mu\ell\ \ell^{-1}$ SO_2 for three hours especially when incubated in deionized water. Incubation in a 0.2 mM $CaSO_4$ solution afforded some protection to the plant cell membranes. Cultivar Bush Blue Lake had significantly higher ion leakage from plants exposed to SO_2, but leakage was greatly reduced when plants were incubated in $CaSO_4$ as compared to those in deionized water (Fig. VII.7a,b). Both Black Turtle Soup (Fig. VII.8 a,b) and Spurt (Fig. VII.9a,b) had significantly greater ion leakage immediately after SO_2 fumigation when incubated in deionized water, but not when incubated in 0.2 mM $CaSO_4$.

Further clarification of air pollution induced membrane leakiness was obtained by allowing 24 hours to elapse between fumigation and incubation. Plants were exposed to 0.1 $\mu\ell\ \ell^{-1}$ O_3 and 0.4 $\mu\ell\ \ell^{-1}$ SO_2 alone or in combination. Exposure to either pollutant stimulated ion leakage in cvs. Bush Blue Lake and Black Turtle Soup (Figs. VII.10-13), but not in cvs. Spurt and French's Horticultural (data not shown). Combined fumigations of O_3 and SO_2 resulted in increased ion leakage from all cultivars (Figs. VII.14-17). The cultivar Bush Blue Lake had similar ion leakage when exposed to SO_2+O_3 as when exposed to O_3 alone (Figs. VII.10,14). Black Turtle Soup had enhanced leakage when exposed to both SO_2 and O_3 compared to exposure to SO_2 or O_3 alone (Figs. VII.12,13,15). As was the case in the previous set of experiments, incubation in $CaSO_4$ reduced ion leakage compared to leakage from leaf discs incubated in deionized water.

Beckerson and Hofstra (1980) investigated the effects of O_3 and SO_2, alone or in combination on ion leakage from leaf strips of soybean, white bean, radish, and cucumber. Both soybean and white bean had increased ion leakage when exposed to O_3 (0.15 $\mu\ell\ \ell^{-1}$, six hours for five consecutive days) but exposure to O_3 and SO_2 combined resulted in an antagonistic or less than additive effect on ion leakage. They suggest that this may be due to the ability of SO_3^{2-} to seal membrane leaks (see Puth and Lüttge, 1973). Radish and cucumber exhibited synergistic responses when exposed to SO_2 and O_3 in combination.

b) Field Study

Field studies were initiated to analyze the growth (Chapter VI) and physiological responses of bean plants, cv. Bush Blue Lake, to long-term, low-level ozone exposure at different soil water regimes. This experiment was not repeated and the physiological parameters measured had a large amount of variability. Measurements were taken at three growth stages: pre-flowering, flowering, and mature pods.

Stomatal conductance was measured during the fumigation period. There were no apparent differences in stomatal conductance between the control and fumigated chamber plants. In all cases, the mean stomatal conductances were higher under optimal soil moisture conditions than under water-stressed conditions, as expected. The large amount of

variability present may have been caused by a number of factors such as non-uniform soil water distribution, differential air flow within the plant canopy, and canopy characteristics resulting in different leaf angles and transpiration. Increasing the number of measurements per treatment from eight to twelve did not appear to have a beneficial effect on the variability of conductance. Stomatal conductance of field chamber-grown bean plants at pre-bloom and flowering were very similar (Figs. VII.18,19); only plants grown at optimal soil water conditions at the time of the final harvest tended to diverge (Fig. VII.20). This result is somewhat suspect, though, due to the visible damage that was apparent for fumigated plants. Plants grown under ambient conditions appeared to have higher stomatal conductances relative to those grown within the field chambers (Figs. VII.18-20). This may be related to the possibility of greater evaporation and transpiration within the chambers because of continuous flow of air, unlike at ambient conditions.

No conclusive results were obtained from the analysis of leaf water potential data (Fig. VII.21) except at the pre-bloom stage. It appears that plants fumigated with ozone had a more negative water potential than those exposed to filtered air. Differences were not nearly as pronounced at the two subsequent harvests even though visible injury appeared significant in fumigated plants.

Ion leakage from bean leaf discs is illustrated in Figs. VII.22-24. Again, variability of results statistically prevents clear distinctions. This variability was not reduced even though replicates were increased from six to ten during the course of the investigation. At the pre-bloom stage, bean plants grown under dry conditions appeared to leak at a greater rate than those of plants grown at optimal soil water conditions (Fig. VII.22). However, the treatments within the soil-water conditions were indistinguishable. Ion leakage from leaf discs of plants harvested during the flowering stage tended to separate out more. Plants exposed to ozone had a higher percent leakage, particularly those grown under drier conditions (Fig. VII.23). Whether or not this suggests an overriding effect of water stress is subject to further investigation. Another possibility is acute damage from ozone exposure resulting in a general breakdown of

leaf compartmentation. Percent ion leakage increased dramatically for chamber-grown plants at the final harvest (Fig. VII.24). This may have been due to the earlier maturity of the chamber-grown plants and the onset of senescence.

Potassium leakage from leaf discs was somewhat less in those plants grown under optimal water conditions at the pre-bloom stage for all three treatments and for ambient and filtered-air grown plants at flowering (Figs. VII.25,26). The decrease was not as pronounced in plants exposed to ozone at either harvest suggesting some interaction of soil-water and ozone exposure.

This field experiment resulted in very variable results and should be repeated. The air flow characteristics of field chambers may have effects upon water loss characteristics of both soil and plants, thus complicating the interpretation of ozone-soil water interactions.

5. Summary

a) Growth Chamber

Low-level fumigation with ozone caused stomatal opening in the two ozone-sensitive bean cultivars but not in the two resistant ones. Ozone sensitivity in bean cultivars therefore appears to be related to high rates of ozone uptake. The effects of SO_2 on these bean cultivars had not been studied before. Low levels of SO_2 exposure did not cause visible injury; the SO_2 effects on stomatal conductance were not correlated with differential SO_2 sensitivity.

Ion leakage from leaf discs determined immediately upon exposure to ozone was not correlated with differential ozone sensitivity in the four bean cultivars. Leakage was stimulated by SO_2, and the addition of Ca ions protected the leaf membranes somewhat from SO_2-stimulated leakage. Ion leakage was also determined 24 hours after termination of air pollutant exposure; there was no correlation between the effects of ozone and SO_2, applied singly or in combination, on leakage and differential cultivar sensitivity. Ozone in combination with SO_2 stimulated leakage from all four cultivars; the addition of Ca ions showed some protection.

b) Field Study

The ozone-sensitive bean cultivar "Bush Blue Lake" was exposed to long-term, low-level ozone (0.07 to 0.1 $\mu\ell\ \ell^{-1}$ O_3 for 5 hours daily) in field chambers at two soil-moisture regimes. At pre-flowering, flowering, and plant maturity the three physiological parameters, stomatal conductance, leaf water potential, and ion leakage from leaf discs, were determined. The plant data from low-level ozone exposure were compared with those from controls (plants grown in field chamber supplied with filtered air) and from plants grown in the field at ambient atmospheric conditions.

The data showed substantial variability. Ozone treatment had no significant effect on stomatal conductance relative to the control, but stomatal conductance was higher in plants grown under well-watered conditions than in water-stressed plants. At the pre-flowering stage, leaf water potential was lower (more negative) in ozone-treated plants relative to the controls, at both low and high soil moisture conditions. The decrease in leaf water potential caused by ozone may be an indicator of air pollution stress in this plant. There was no significant effect of ozone on ion leakage from leaf discs at the pre-flowering stage, but at flowering ozone tended to stimulate ion leakage, particularly in water-stressed plants (it is not clear whether ozone or water stress was the overriding factor in this case).

In order to more fully evaluate the effects of ozone on the measured physiological parameters, we suggest that this field study be repeated and expanded.

6. References

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Table VII.1 Effects of 2.5 hours of 0.1 $\mu\ell\ \ell^{-1}$ O_3 on the stomatal conductance of four bean cultivars. Each value is the mean of four measurements made on the lower side of the primary leaves of four plants. Asterisk denotes significant difference at the 5% level.

Cultivar	Δ $CmSec^{-1}$	% of Control
O_3-sensitive		
Bush Blue Lake	+ 0.33*	206
Spurt	+ 0.15*	129
O_3-resistant		
Black Turtle Soup	- 0.02	94
French's Hort.	+ 0.08	133[a]

[a]This number appears high because of the low stomatal conductance before fumigation. The actual change in conductance (0.08 cm sec^{-1}) was small.

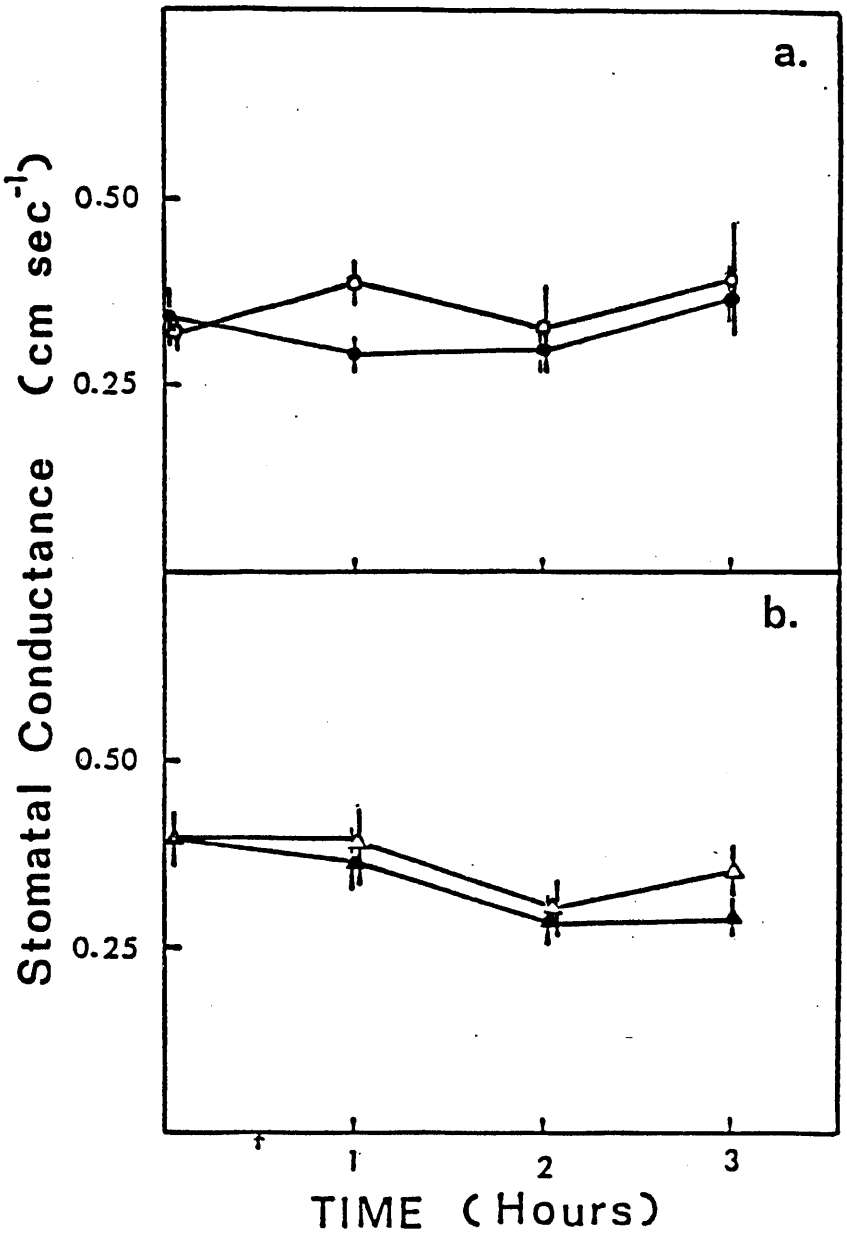

Figure VII.1 Effects of low-level SO_2 (0.4 $\mu\ell$ ℓ^{-1}, 2.5 hours) on bean stomatal conductance. a. Bush Blue Lake 290 (O_3-sensitive). b. Black Turtle Soup (O_3-resistant). Arrow denotes beginning of fumigation. Each datum is the mean of eight replicate samples. Open symbols represent controls. Closed symbols represent SO_2 treatment.

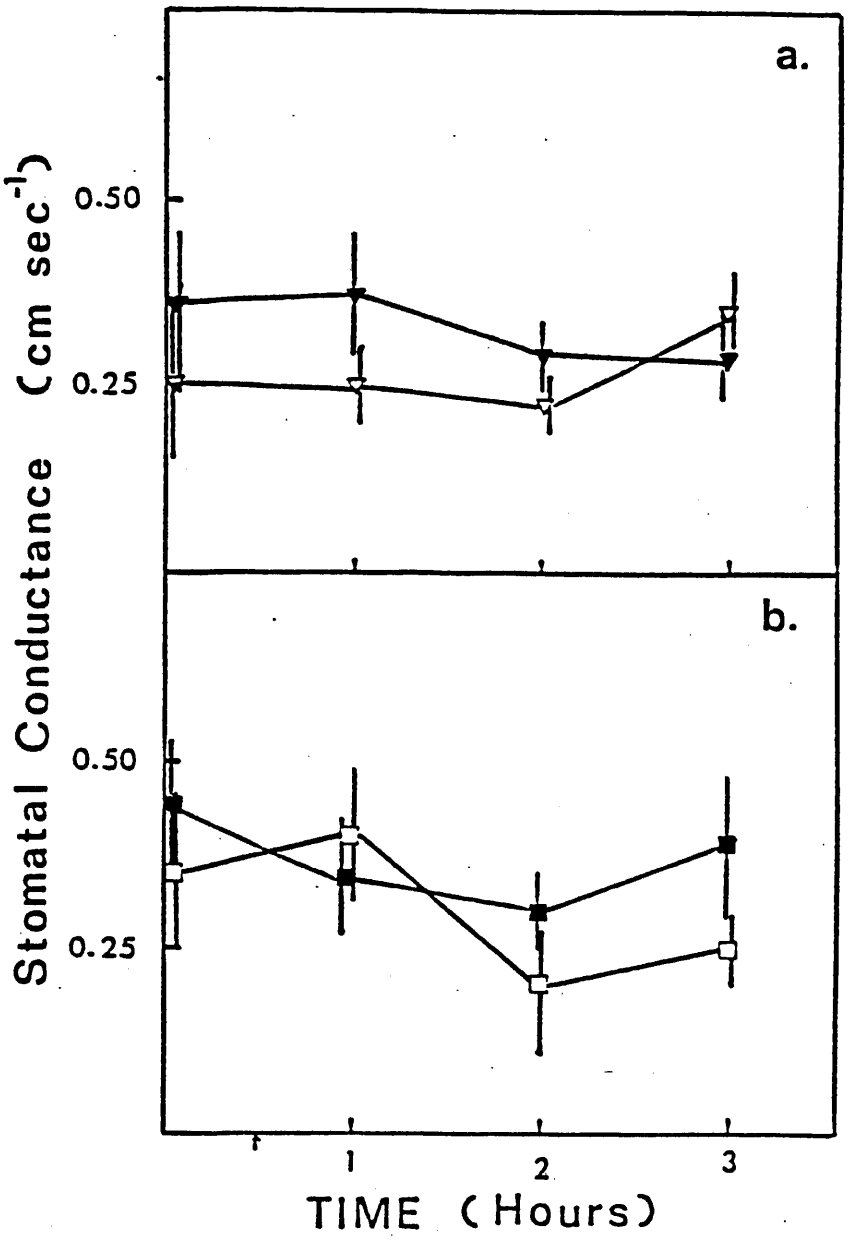

Figure VII.2 Effects of low-level SO_2 (0.4 $\mu\ell\ \ell^{-1}$, 2.5 hours) on stomatal conductance of beans. a. French's Horticultural (O_3-resistant). b. Spurt (O_3-sensitive). Arrow denotes beginning of fumigation. Each datum is the mean of eight replicate samples. Open symbols represent controls. Closed symbols are SO_2 treatment.

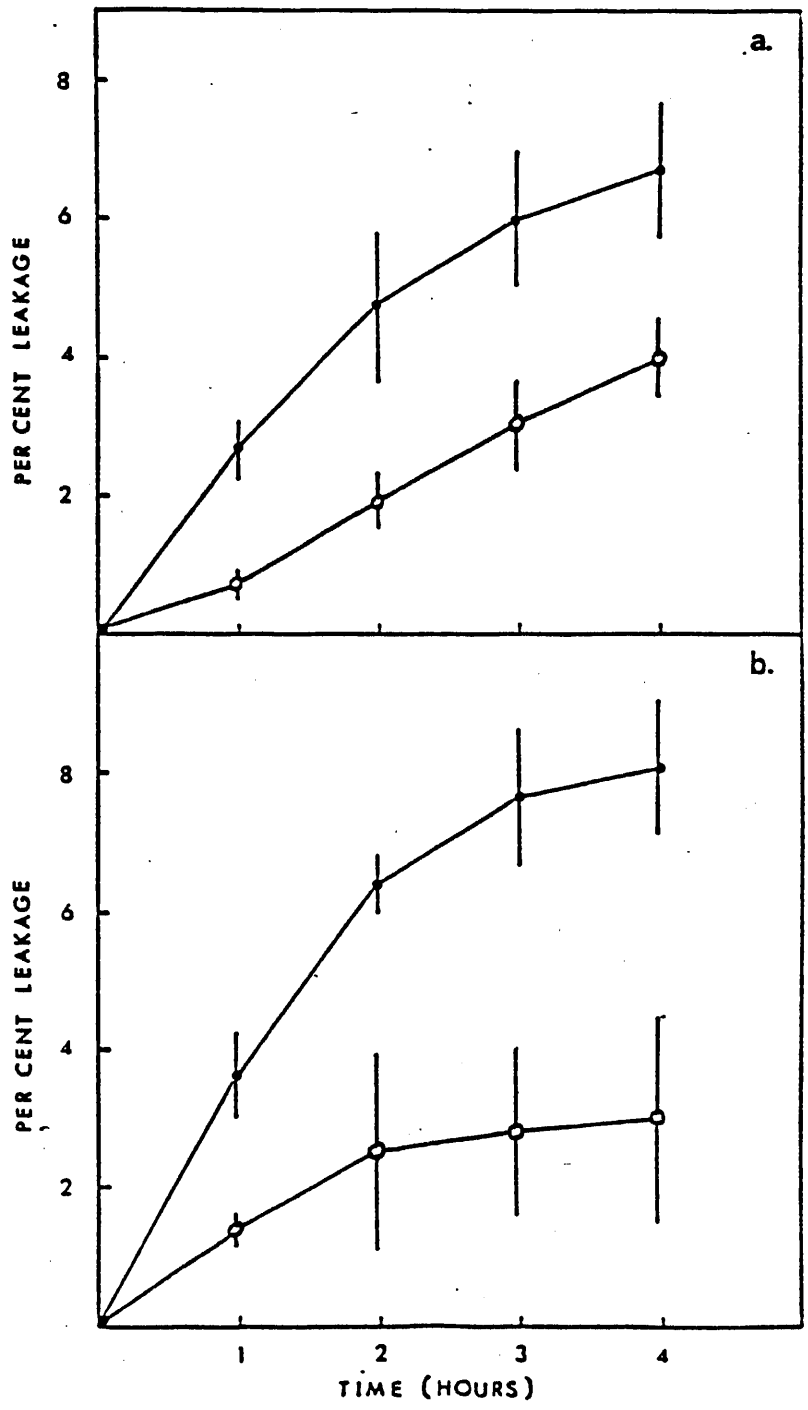

Figure VII.3 Effects of low-level O_3 (0.1 $\mu\ell\ \ell^{-1}$, 3 hours) on ion leakage from bean, cultivar Bush Blue Lake 290. Each datum is the mean of three replicate samples. Time axis indicates leaf disc incubation period. Open circles (0) are controls and closed circles (●) are O_3 treatment. a. Leaf discs incubated in deionized, distilled water. b. Leaf discs incubated in 0.2 mM $CaSO_4$.

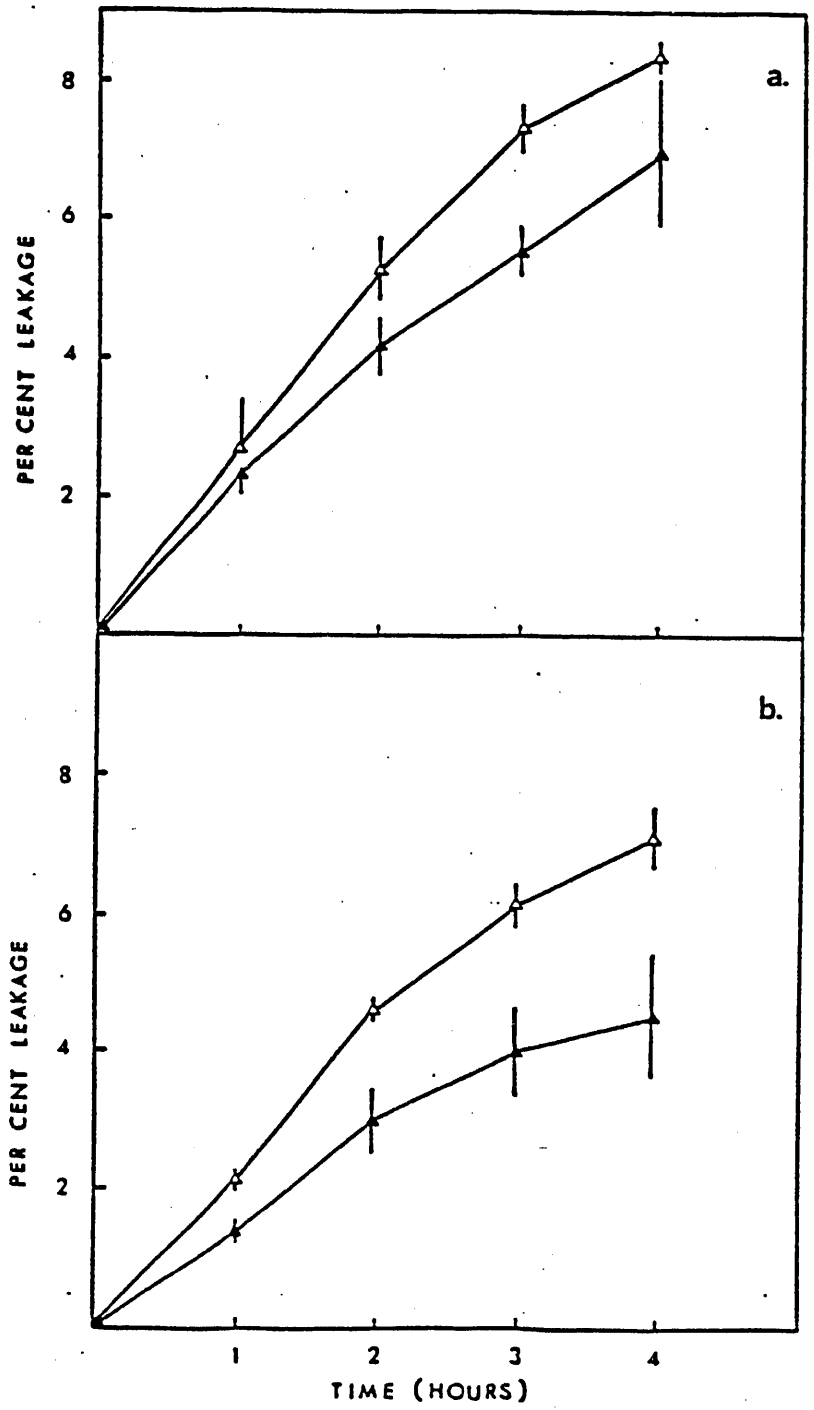

Figure VII.4 Effects of low-level O_3 (0.1 $\mu\ell\ \ell^{-1}$, 3 hours) on ion leakage from bean, cultivar Black Turtle Soup. Each datum is the mean of three replicate samples. Open triangles (Δ) represent controls. Closed triangles (▲) represent O_3 treatment. a. Deionized, distilled water incubation. b. 0.2 mM $CaSO_4$ incubation. Time axis indicates leaf disc incubation period.

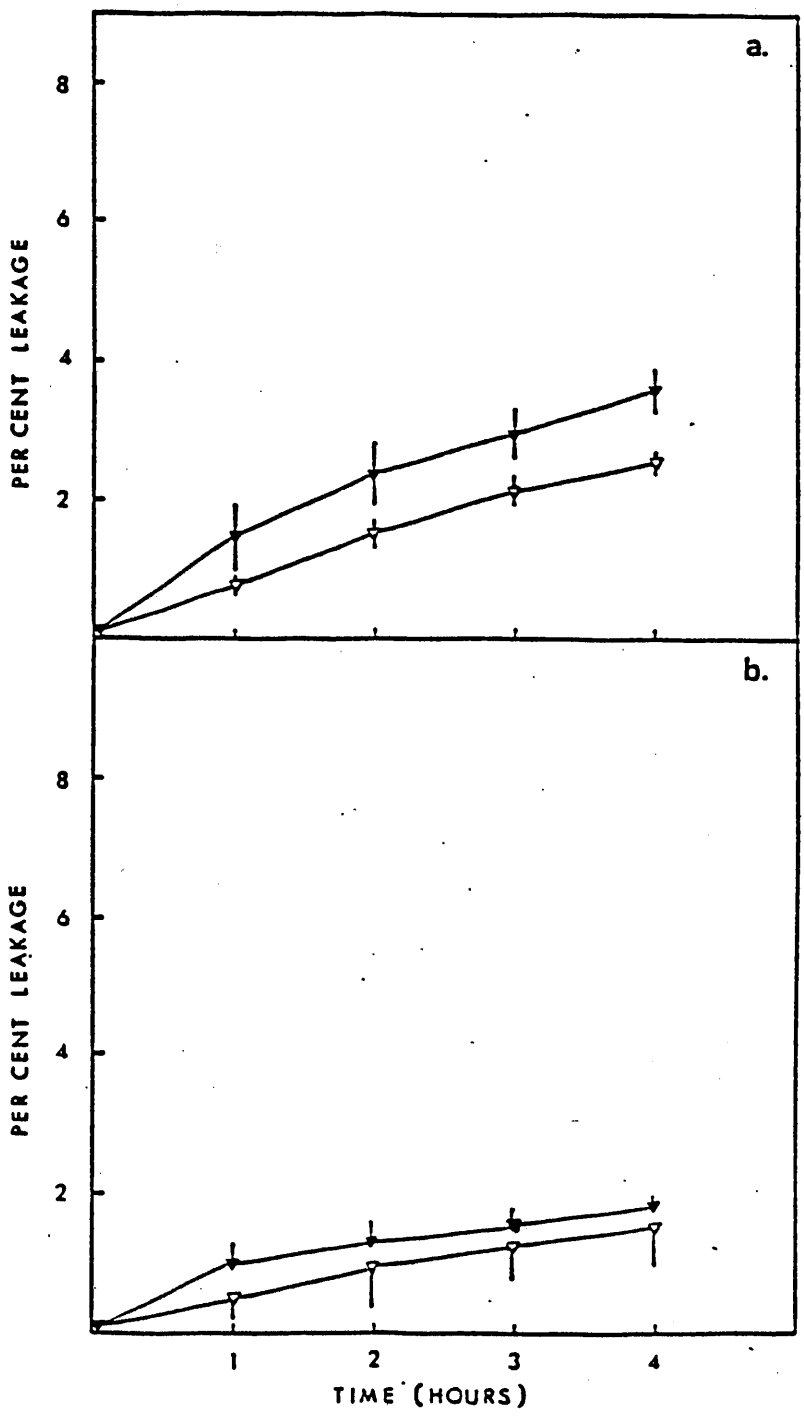

Figure VII.5 Effects of low-level O_3 (0.1 $\mu\ell\ \ell^{-1}$, 3 hours) on ion leakage from bean, cultivar French's Horticultural. Each datum is the mean of three replicate samples. Open inverted triangles (▽) are controls. Closed triangles (▼) are O_3 treatment. a. Deionized, distilled water incubation. b. 0.2 mm $CaSO_4$ incubation. Time axis is incubation period.

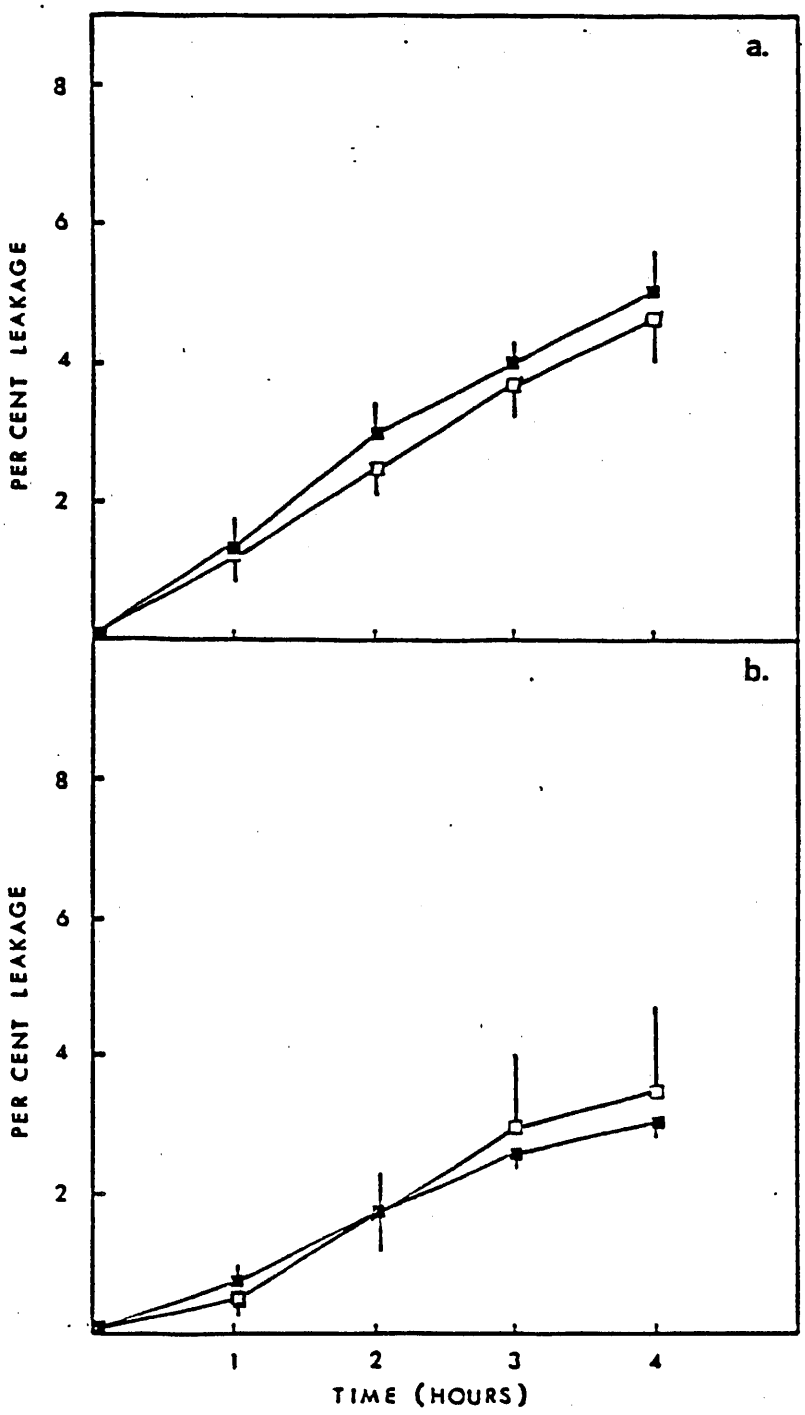

Figure VII.6 Effects of low-level O_3 (0.1 $\mu\ell\ \ell^{-1}$, 3 hours) on ion leakage from bean, cultivar Spurt. Each datum is the mean of three replicate samples. Open squares (□) are controls. Closed squares are O_3 treatment (■). a. Deionized, distilled water incubation. b. 0.2 mM $CaSO_4$ incubation.

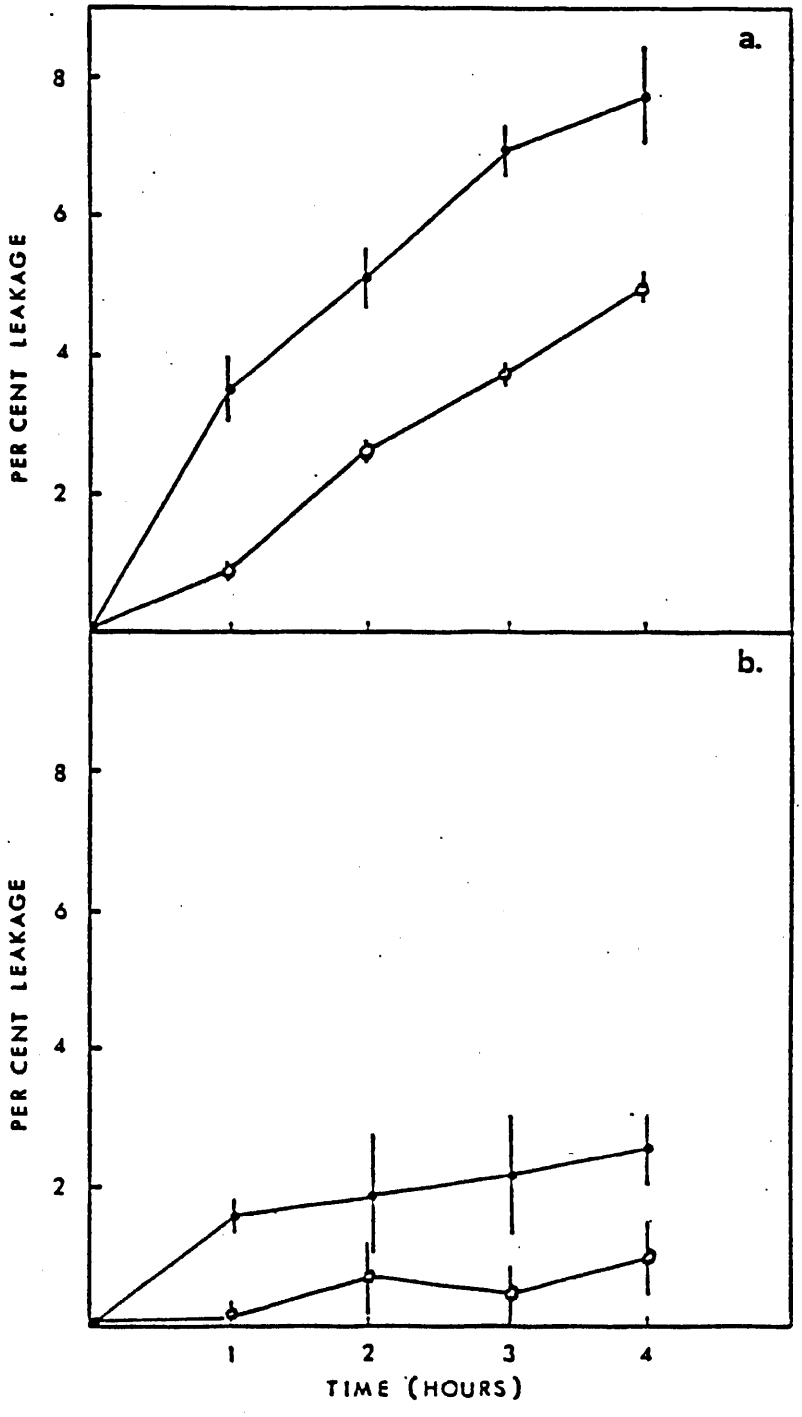

Figure VII.7 Effects of low-level SO_2 (0.4 $\mu\ell\ \ell^{-1}$, 3 hours) on ion leakage from bean, cultivar Bush Blue Lake 290. Each datum is the mean of three replicate samples. Open circle (O) are controls. Closed circles (●) are SO_2 treated. a. Deionized, distilled water incubation. b. 0.2 mM $CaSO_4$ incubation.

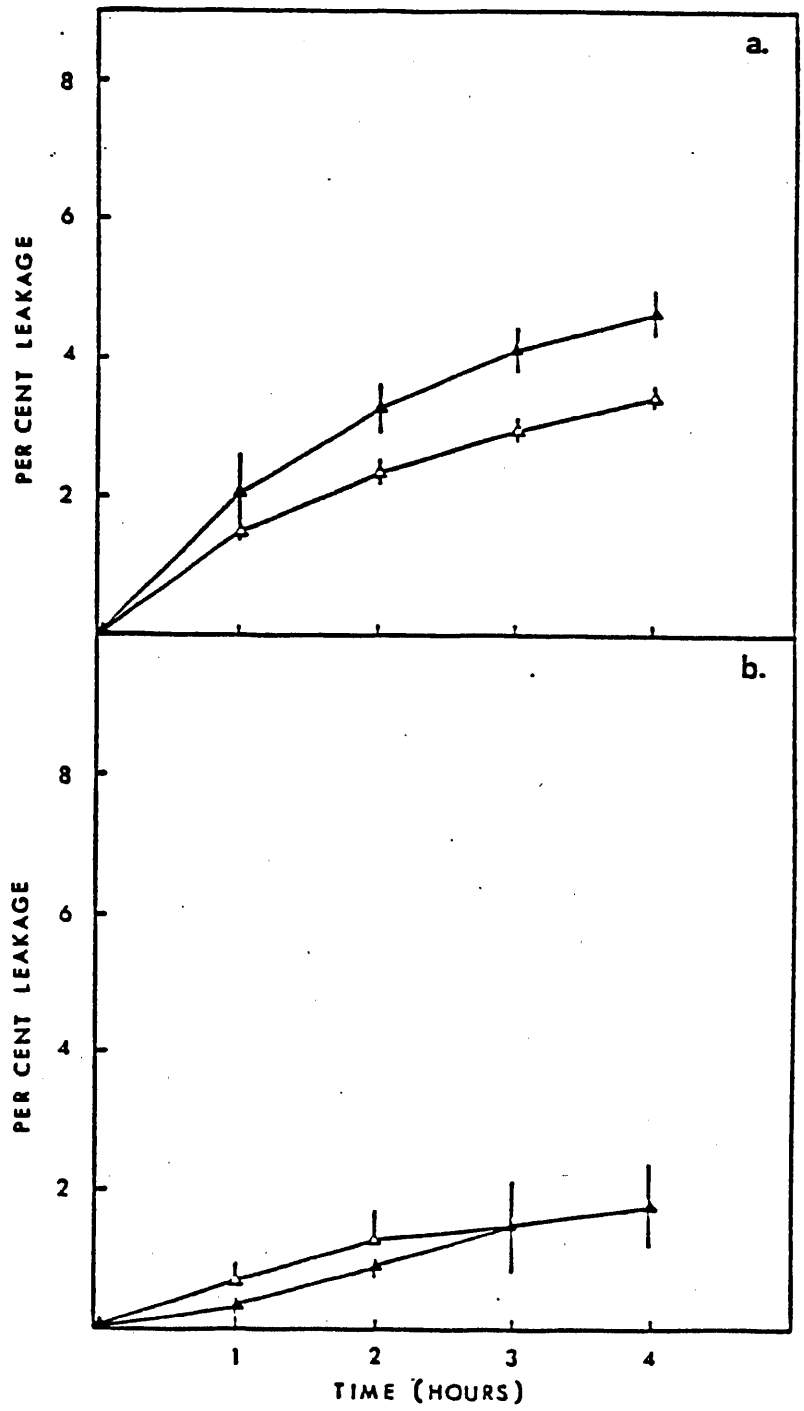

Figure VII.8 Effects of low-level SO_2 (0.4 $\mu\ell\ \ell^{-1}$, 3 hours) on ion leakage from bean, cultivar Black Turtle Soup. Each datum is the mean of three replicate samples. Open triangles (Δ) denote controls. Closed triangles (▲) denote SO_2 treatment. a. Deionized, distilled water incubation. b. 0.2mM $CaSO_4$ incubation.

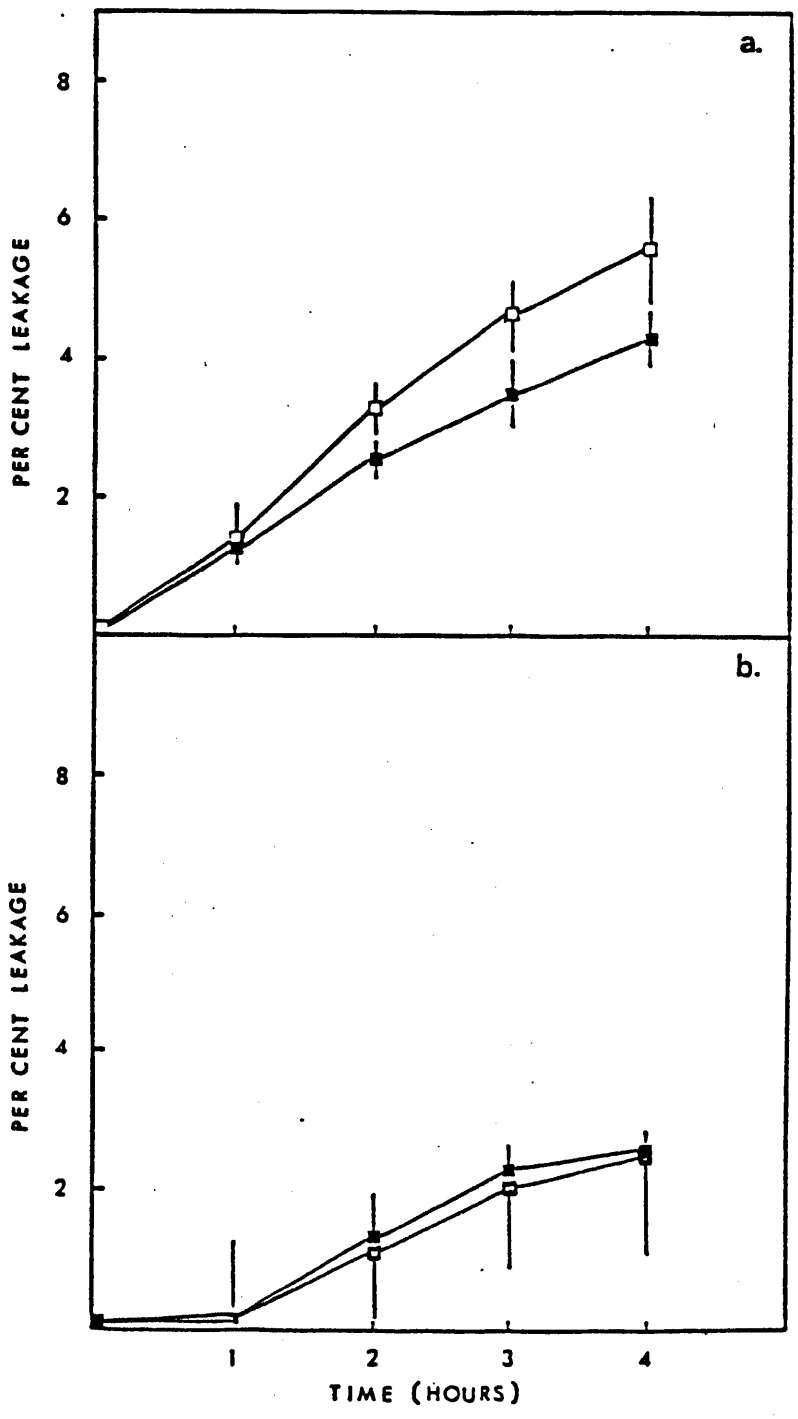

Figure VII.9 Effects of low-level SO_2 (0.4 $\mu\ell\ \ell^{-1}$, 3 hours) on ion leakage from bean, cultivar Spurt. Each datum is the mean of three replicate samples. Open squares (□) are controls. Closed squares (■) are SO_2 treatment. a. Deionized, distilled water incubation. b. 0.2 mM $CaSO_4$ incubation.

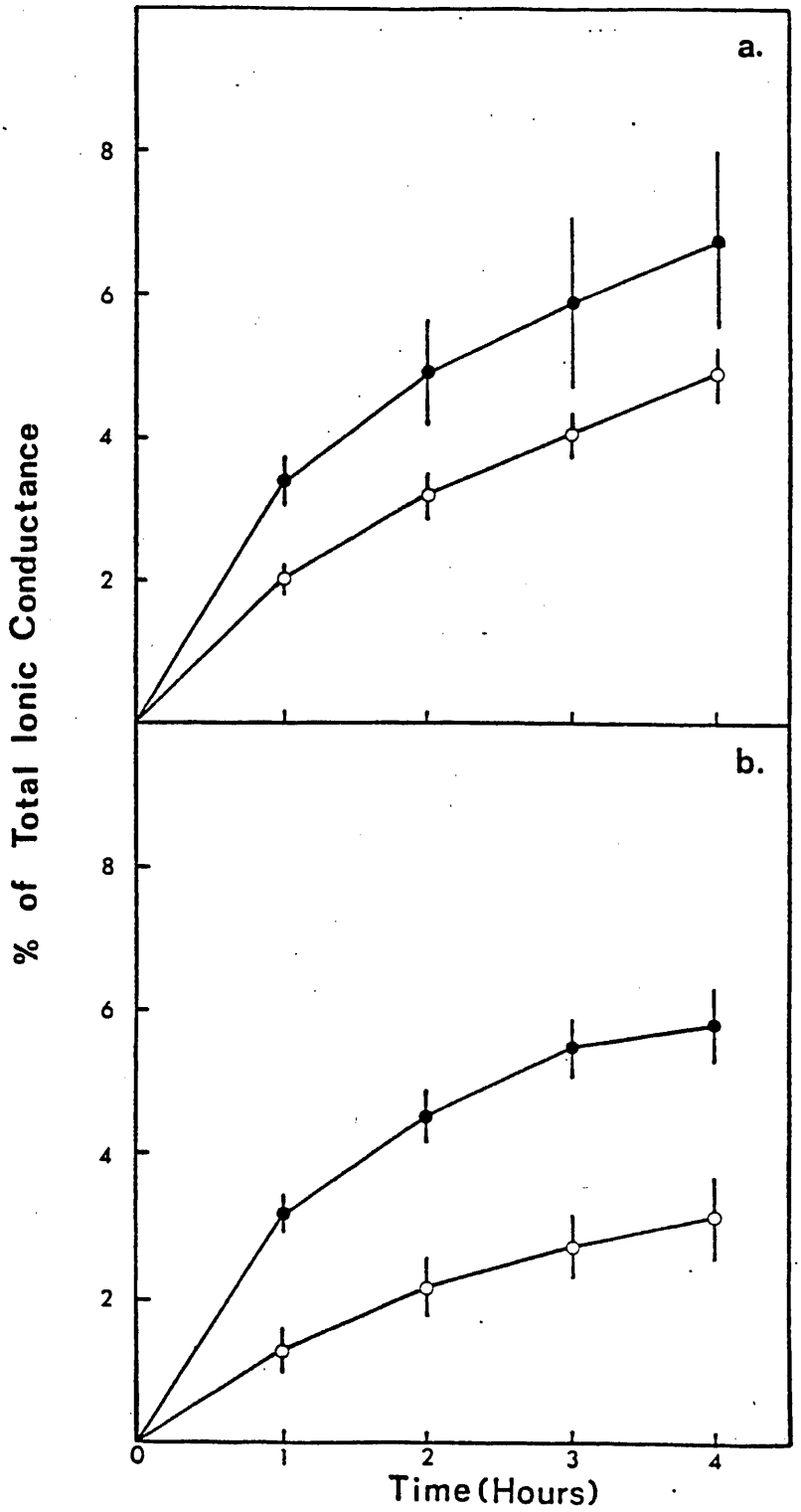

Figure VII.10 a. BBL leaf discs (prepared 24 hours after O_3 exposure) incubated in deionized water (H_2O). b. BBL discs incubated in 0.2 mM $CaSO_4$. Closed symbols represent O_3 treatment; open symbols are controls.

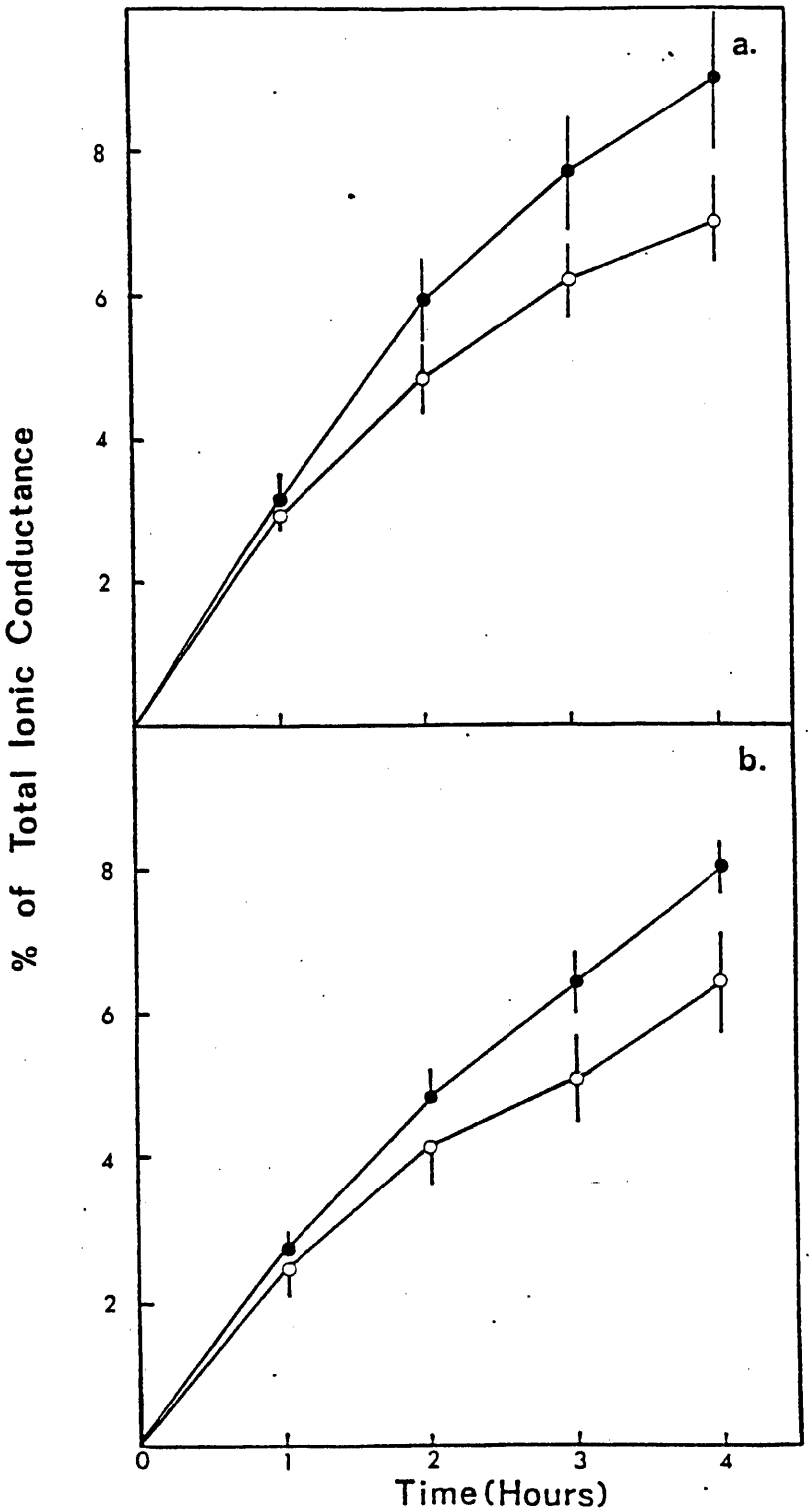

Figure VII.11 a. BBL leaf discs (prepared 24 hours after SO_2 exposure) incubated in H_2O. b. BBL discs incubated in 0.2 mM $CaSO_4$. Closed symbols are SO_2 treatment; open symbols are controls.

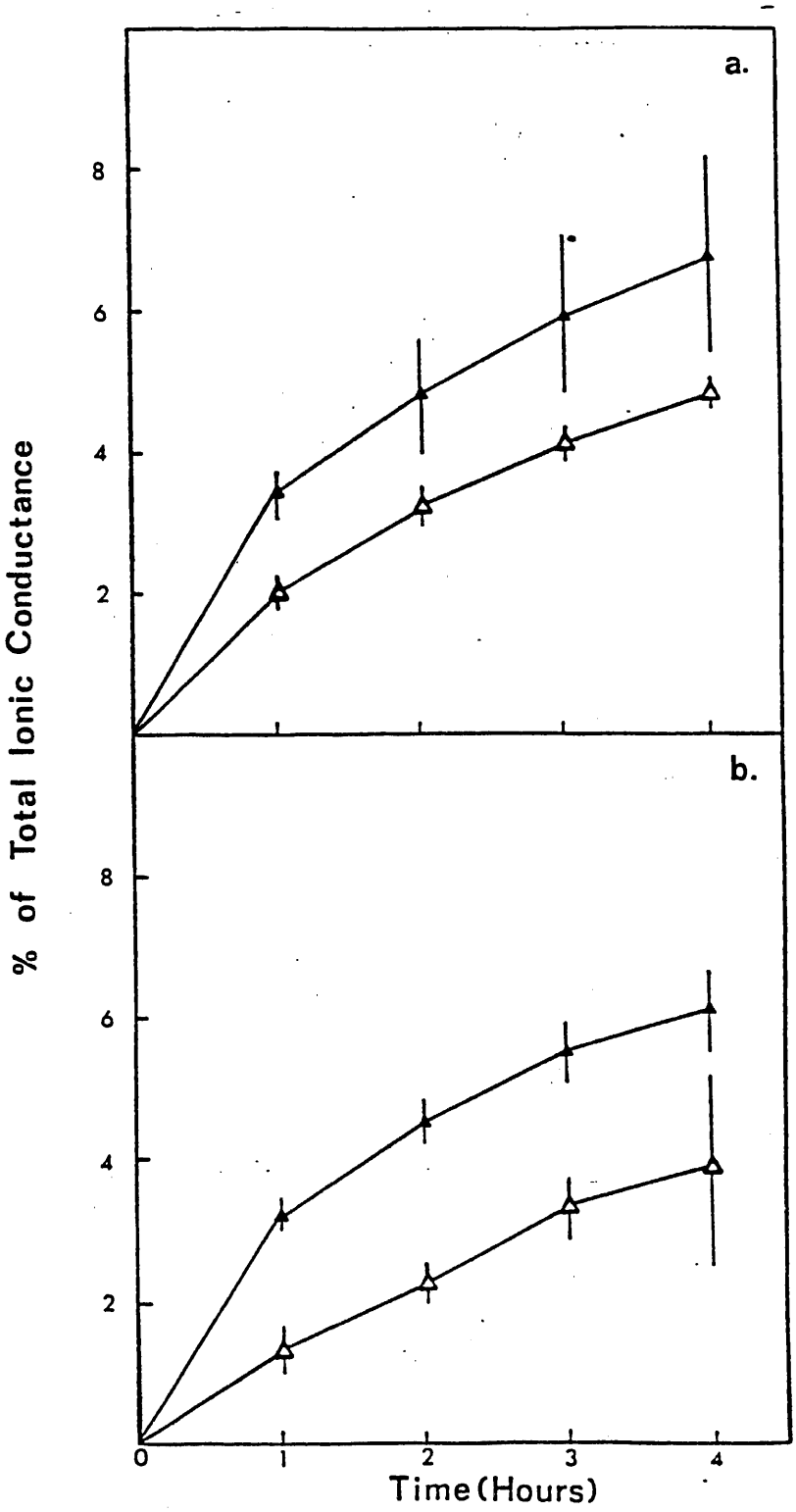

Figure VII.12 a. BTS leaf discs (prepared 24 hours after O_3 treatment) incubated in H_2O. b. BTS discs incubated in 0.2 mM $CaSO_4$. Closed symbols are O_3 treatment; open symbols are controls.

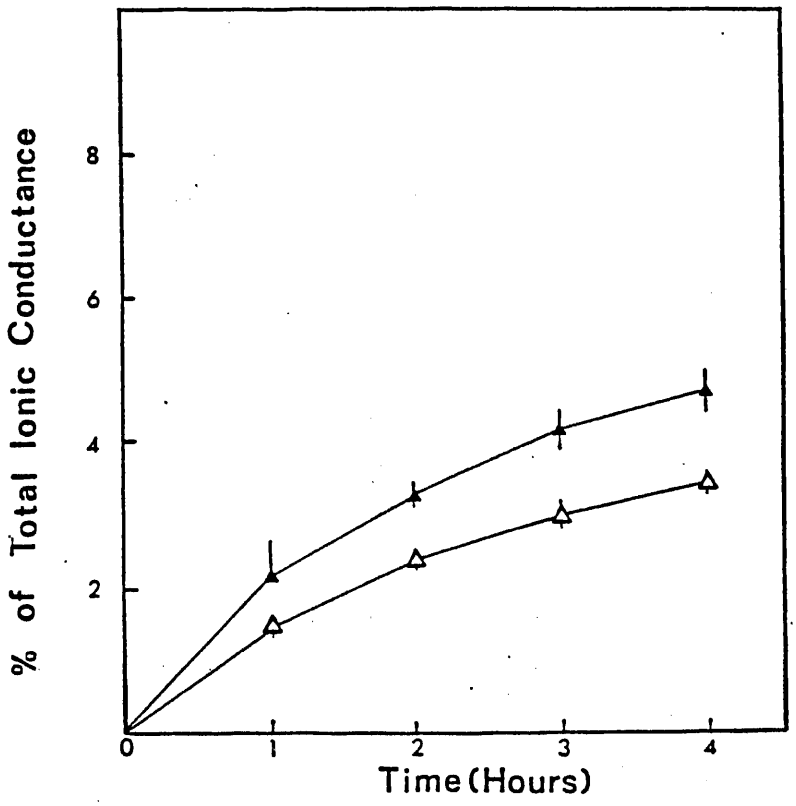

Figure VII.13 BTS leaf discs (prepared 24 hours after SO_2 treatment) incubated in H_2O. Closed symbols are SO_2 treatment; open symbols are controls.

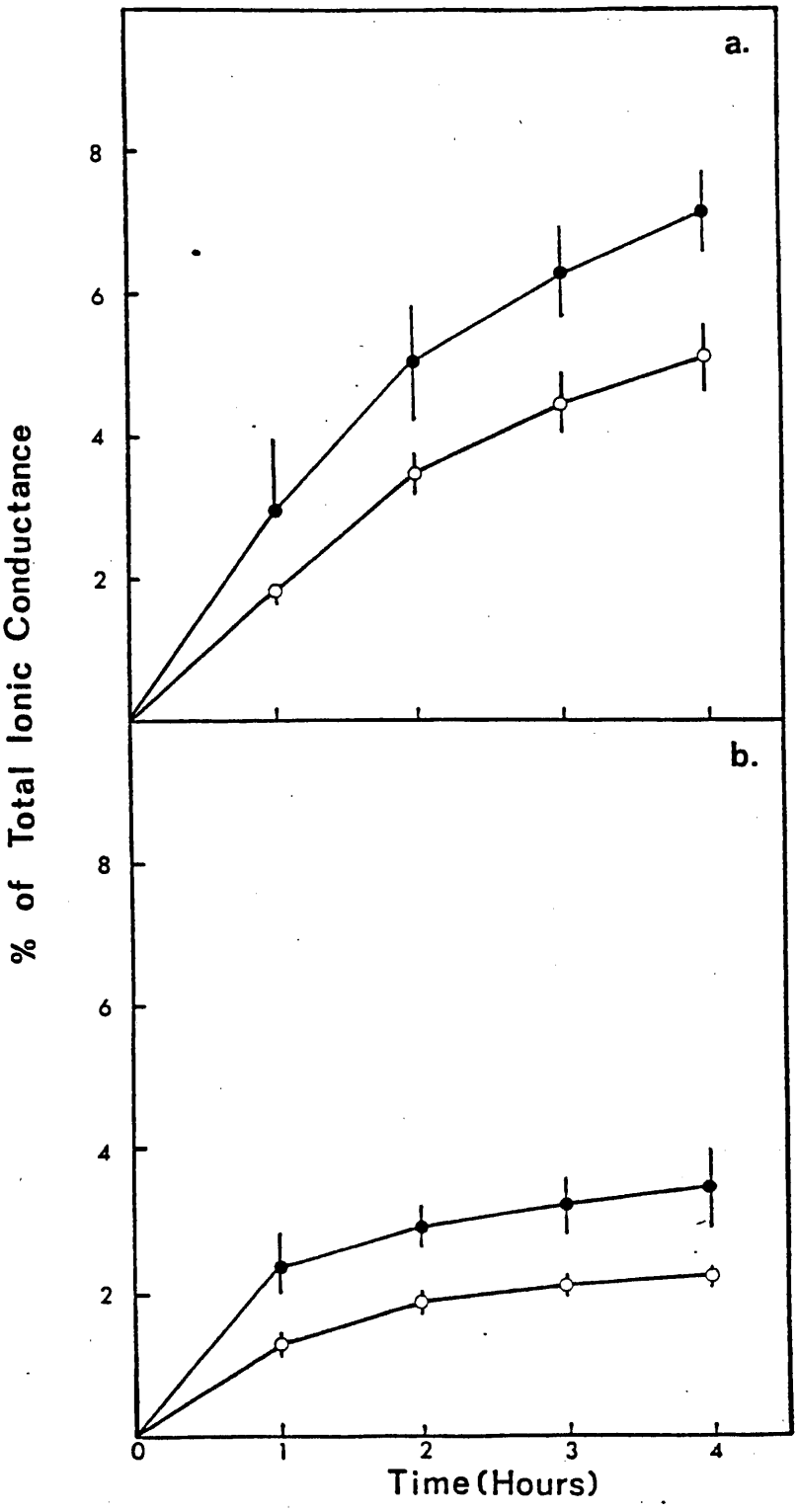

Figure VII.14 a. BBL leaf discs (prepared 24 hours after SO_2xO_3 treatment) incubated in H_2O. b. BBL discs incubated in 0.2 mM $CaSO_4$. Closed symbols are SO_2xO_3 treatment; open symbols are controls.

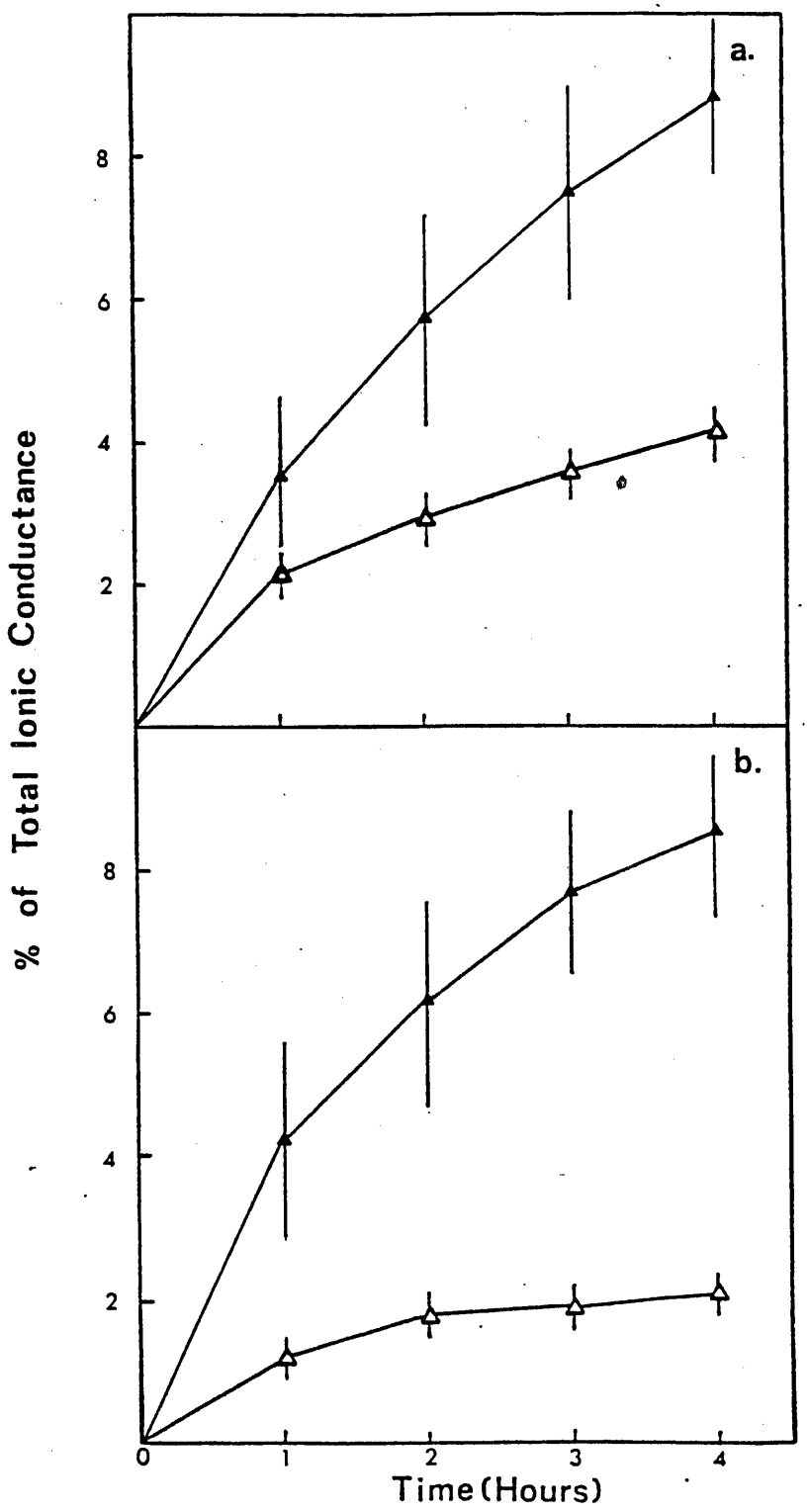

Figure VII.15 a. BTS leaf discs (prepared 24 hours after SO_2xO_3 treatment) incubated in H_2O. b. BTS discs incubated in 0.2 mM $CaSO_4$. Closed symbols are SO_2xO_3 treatment; open symbols are controls.

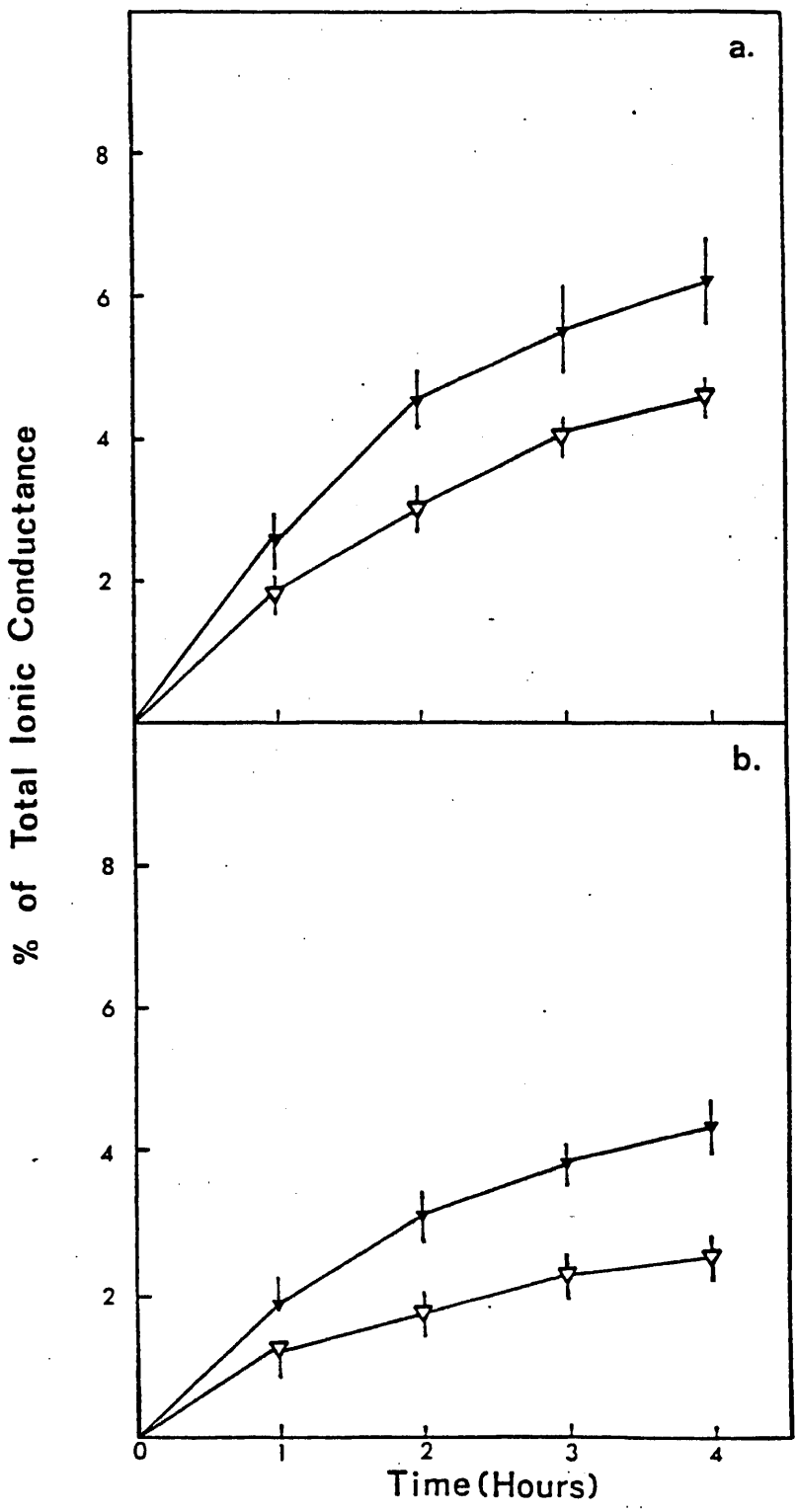

Figure VII.16 a. FH leaf discs (prepared 24 hours after SO_2xO_3 treatment) incubated in H_2O. b. FH discs incubated 0.2 mM $CaSO_4$. Closed symbols are SO_2xO_3 treatment; open symbols are controls.

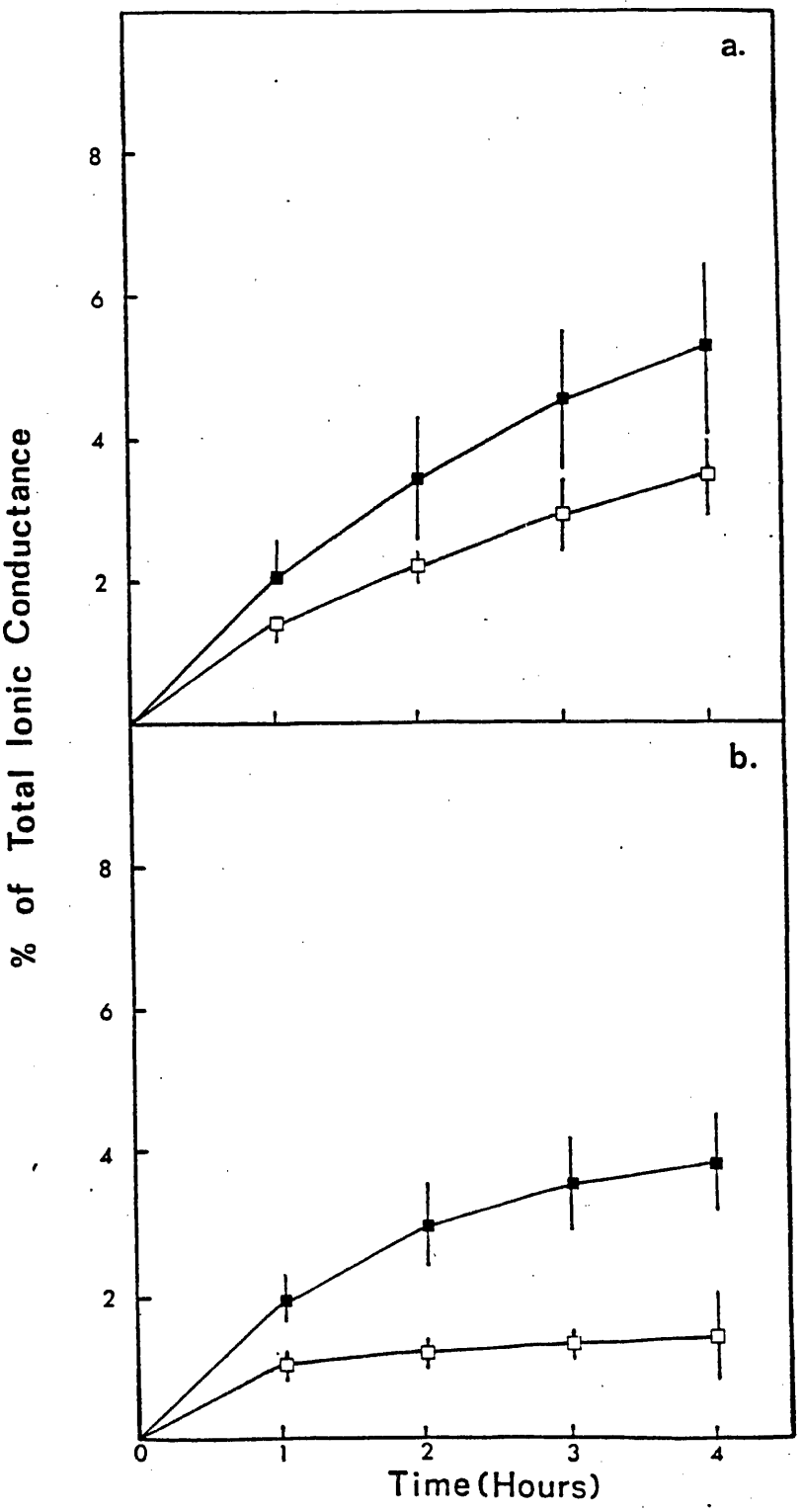

Figure VII.17 a. S leaf discs (prepared 24 hours after SO_2xO_3 treatment) incubated in H_2O. b. S discs incubated in 0.2 mM $CaSO_4$. Closed symbols are SO_2xO_3 treatment; open symbols are controls.

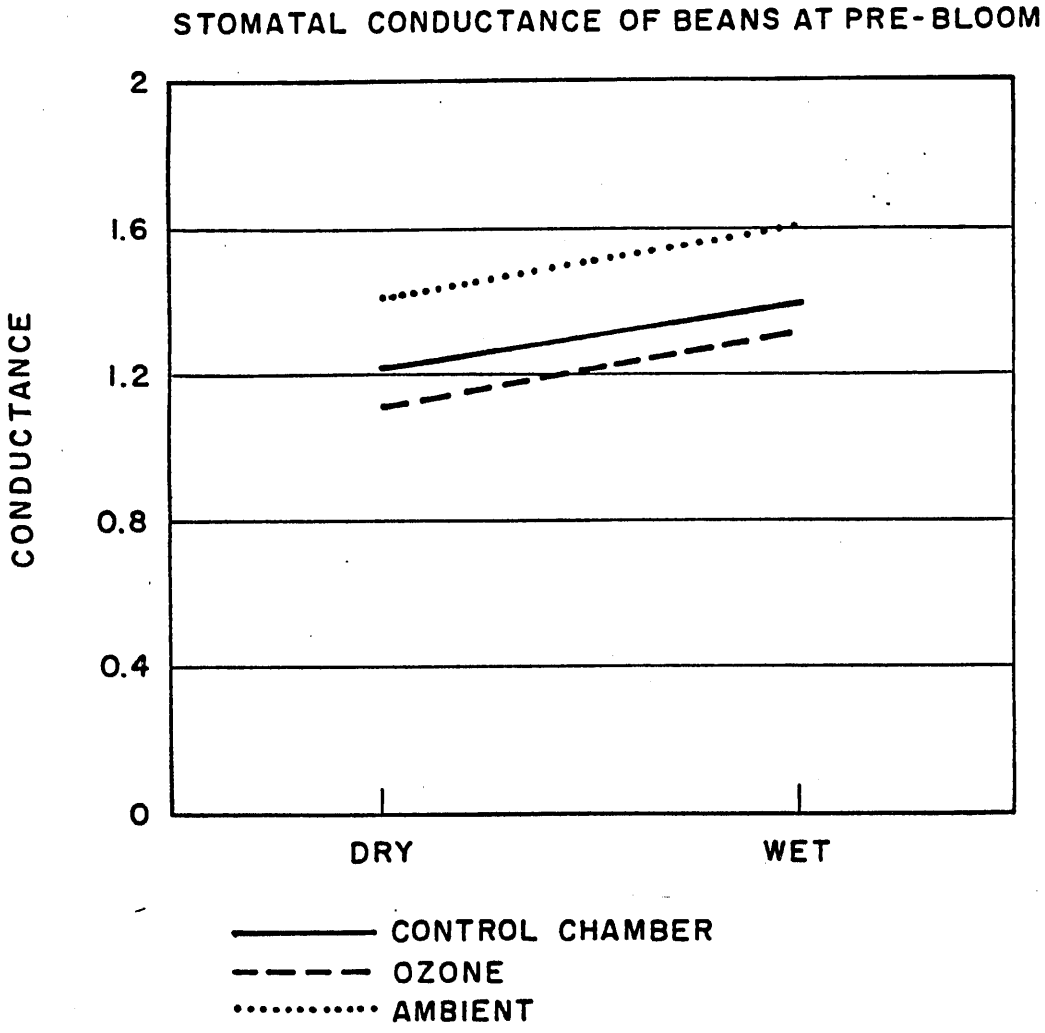

Figure VII.18 Stomatal conductance of bean leaves at the pre-bloom growth stage at two soil-water regimes. Mean of sixteen measurements.

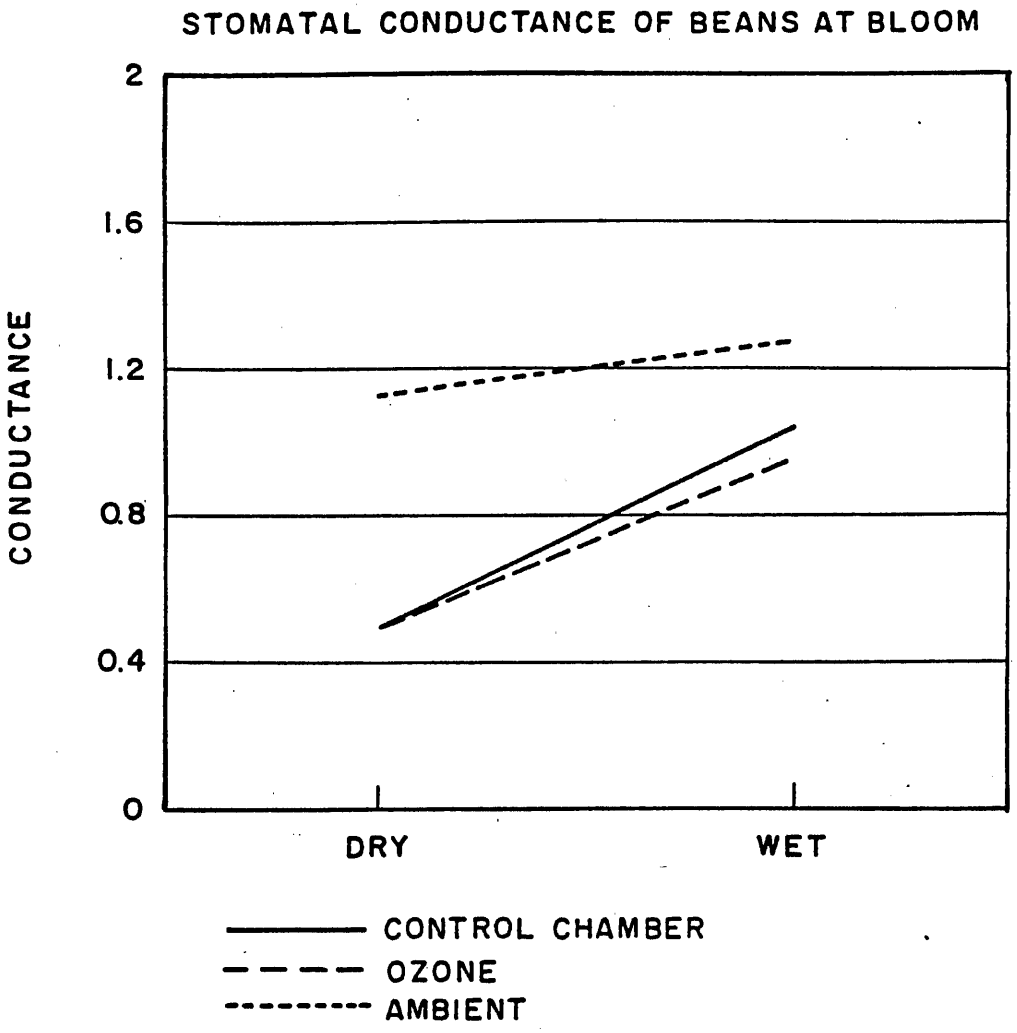

Figure VII.19 Stomatal conductance of bean leaves during flowering at two soil-water regimes. Mean of sixteen measurements.

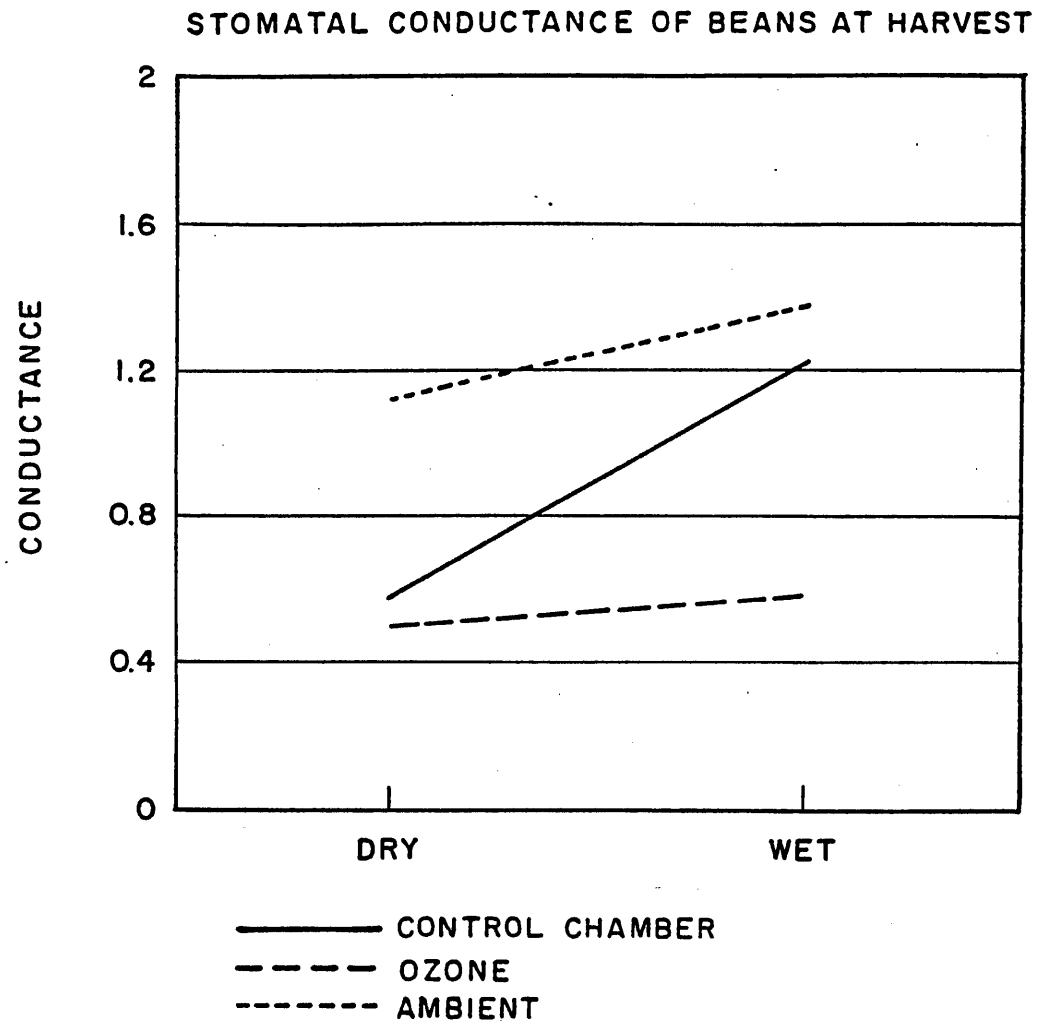

Figure VII.20 Stomatal conductance of bean leaves at final harvest (mature pods) at two soil-water regimes. Mean of twenty-four measurements.

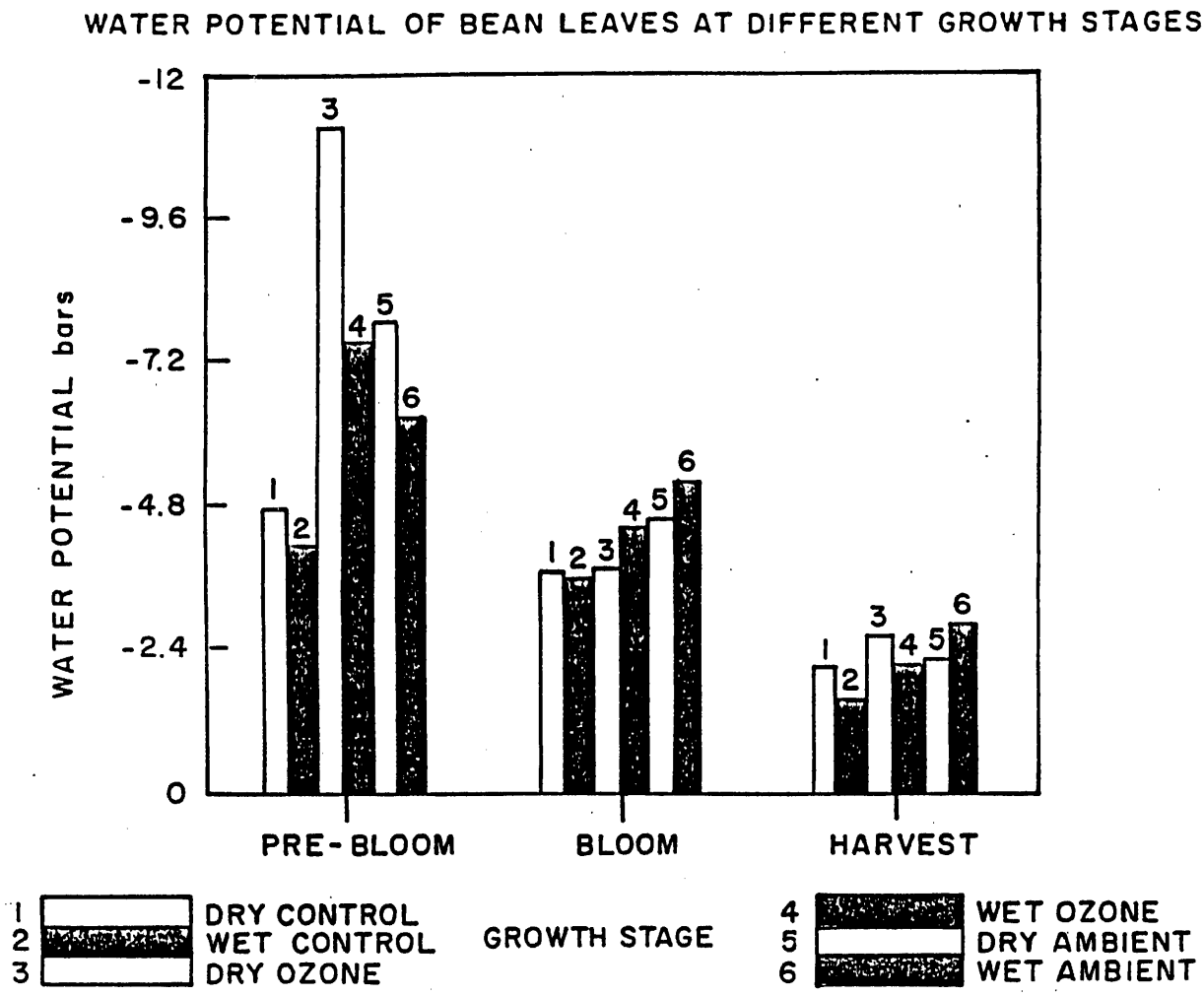

Figure VII.21 Leaf water potential (bars) of bean plants at three growth stages and two soil-water regimes. Mean of eight, eight, and twelve measurements for the three growth stages, respectively.

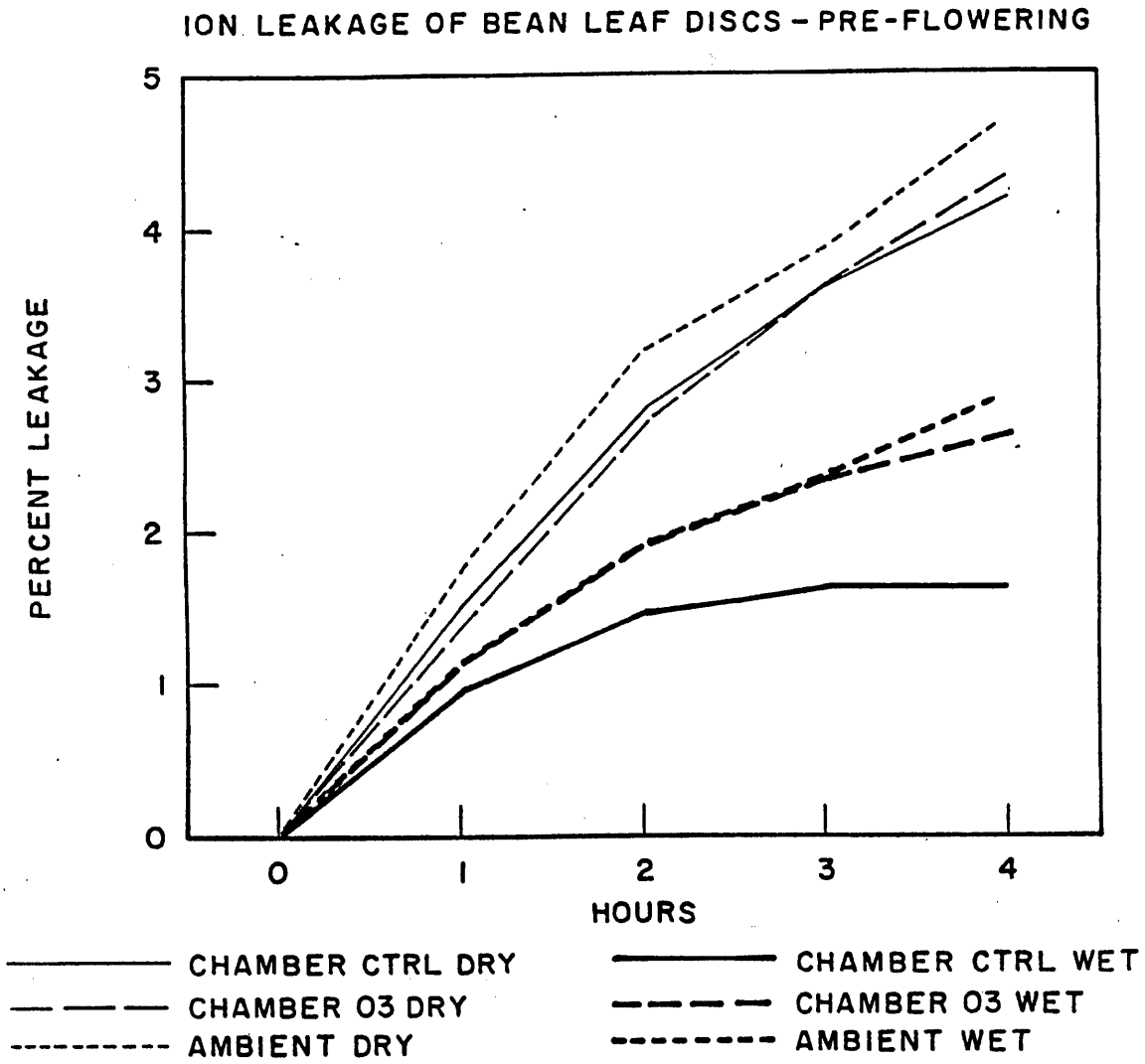

Figure VII.22 Mean percent ion leakage of bean leaf discs at the pre-bloom growth stage at two soil-water regimes. The three curves with the highest leakage are from plants experiencing water stress. Mean of six measurements.

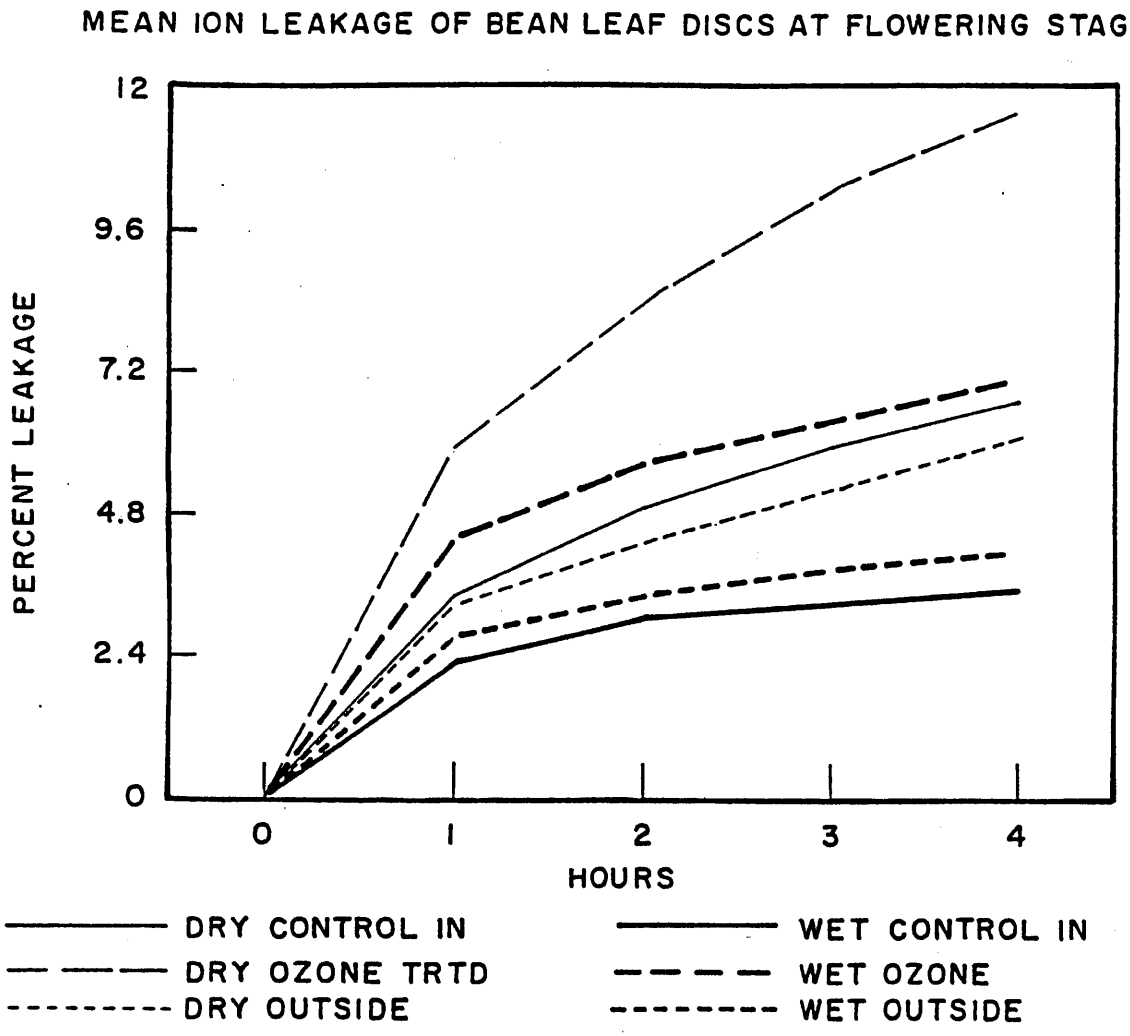

Figure VII.23 Mean percent ion leakage of bean leaf discs at the flowering stage at two soil-water regimes. The upper-most, third, and fourth curves are from plants grown under water stress conditions. Mean of eight measurements.

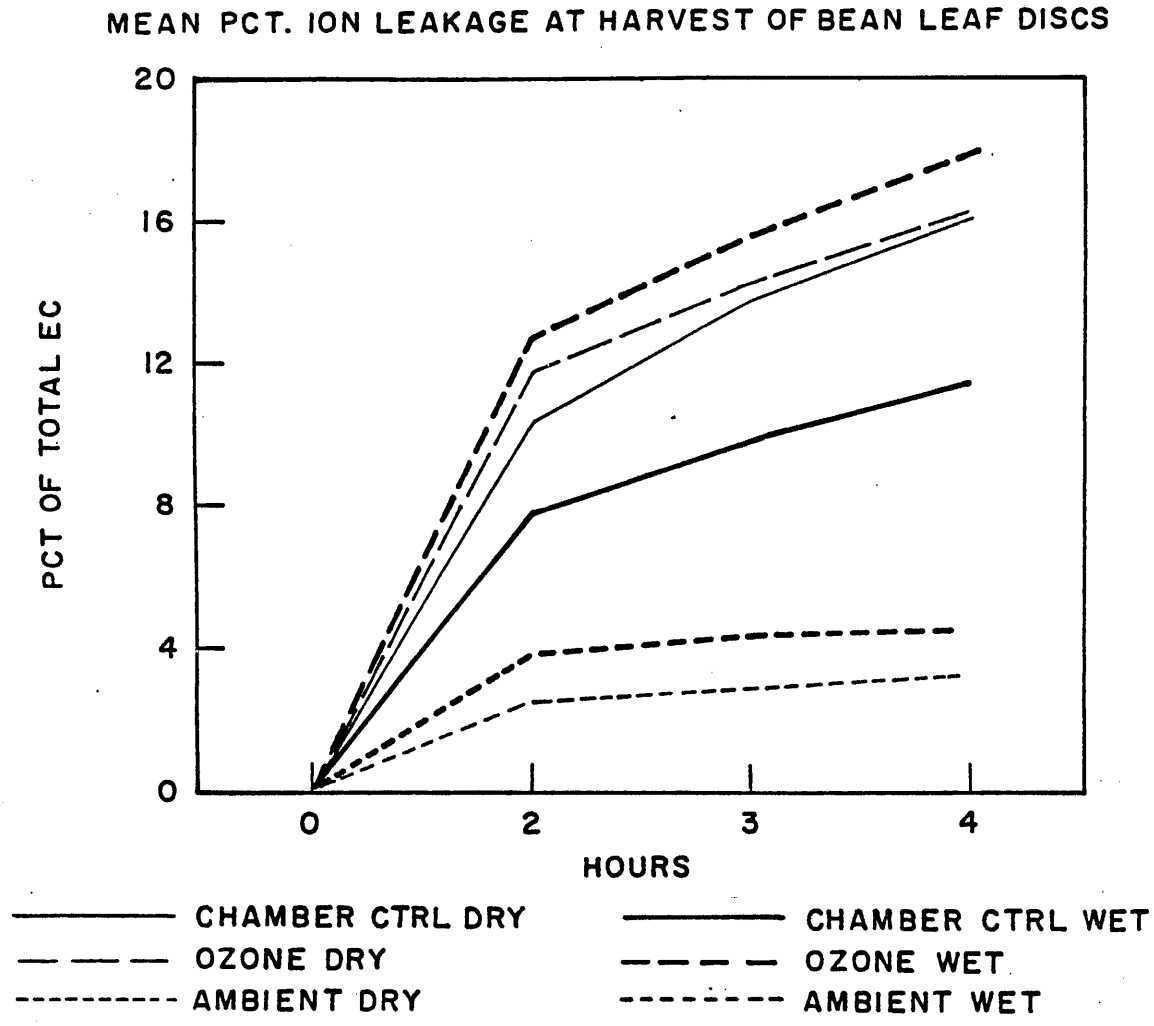

Figure VII.24 Mean percent ion leakage of bean leaf discs at the final harvest (mature pods). The second, third, and sixth lines are from plants grown under water stress conditions. Mean of ten measurements.

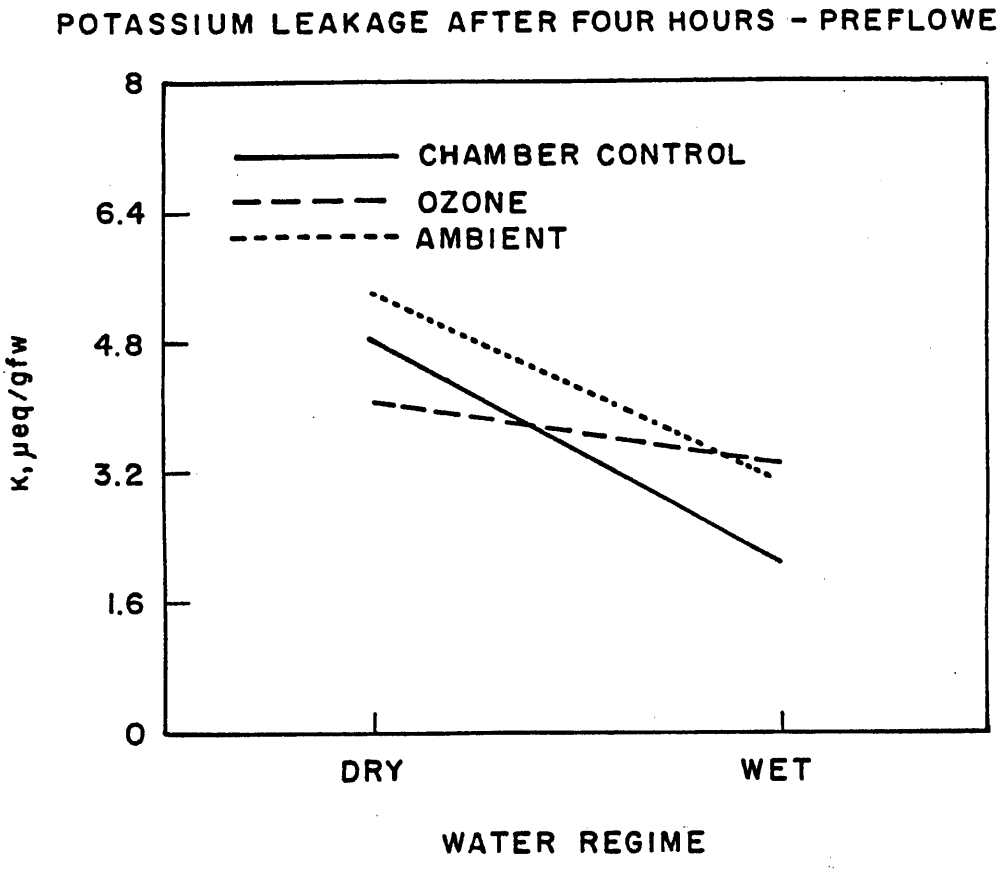

Figure VII.25 Potassium leakage (µeq/gram fresh weight) from bean leaf discs after four hour incubation at the pre-bloom growth stage under two soil-water regimes. Mean of six measurements.

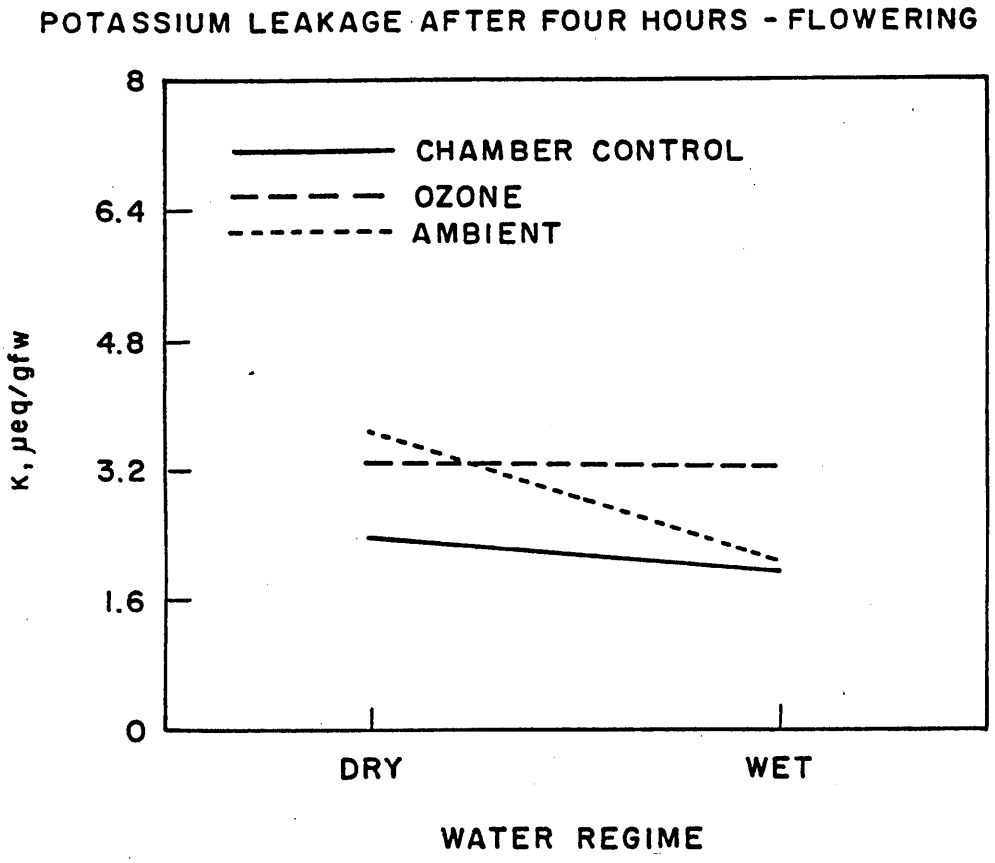

Figure VII.26 Potassium leakage (μeq/gram fresh weight) from bean leaf discs during flowering at two soil-water regimes. Mean of eight measurements.

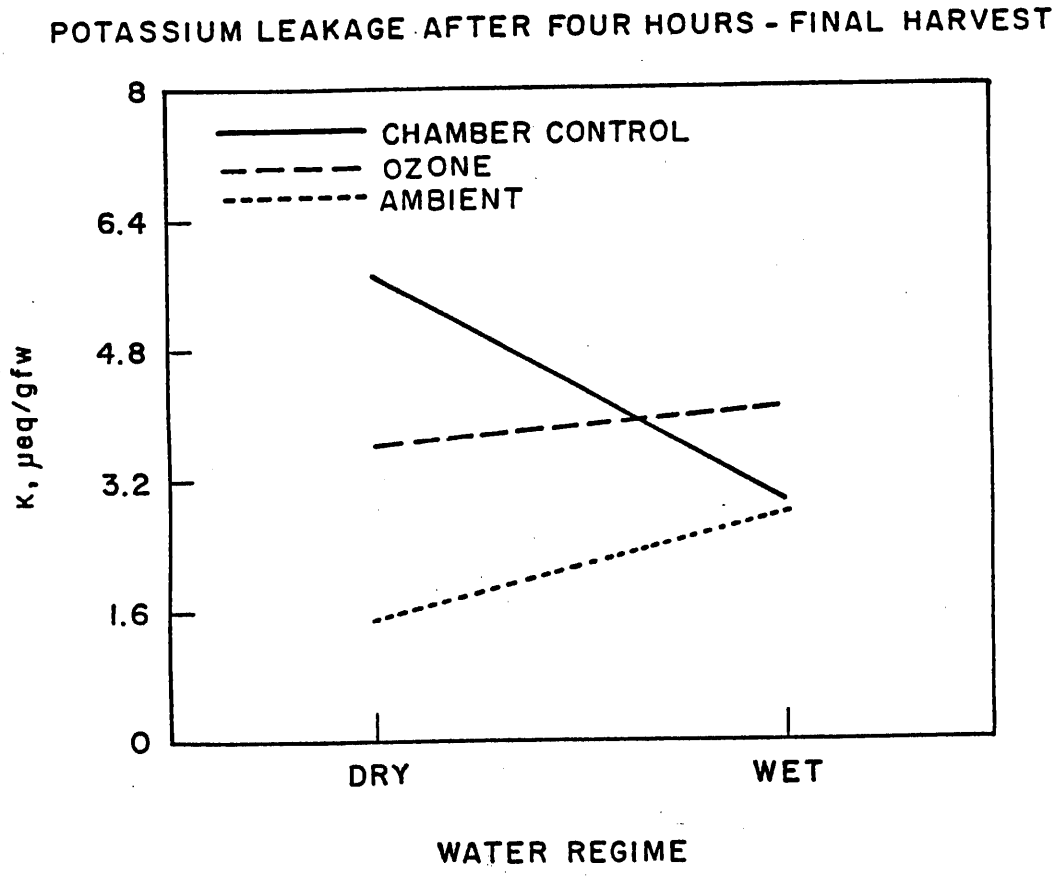

Figure VII.27 Potassium leakage (µeq/gram fresh weight) from bean leaf discs at final harvest (mature pods) at two soil-water regimes. Mean of ten measurements.

10. LIST OF PUBLICATIONS

Peiser, G. D., M. C. Lizada and S. F. Yang. 1982. Sulfite-induced lipid peroxidation in chloroplasts as determined by ethane formation. Plant Physiol. 70:994-998.

We intend to publish all other major results of this comprehensive study in technical journals; manuscripts for these additional publications are in preparation.

Plant Physiol. (1982) 70, 994–998
0032-0889/82/70/0994/05/$00.50/0

Sulfite-Induced Lipid Peroxidation in Chloroplasts as Determined by Ethane Production[1]

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ABSTRACT

Ethane formation, as a measure of lipid peroxidation, was studied in spinach (*Spinacia oleracea* L.) chloroplasts exposed to sulfite. Ethane formation required sulfite and light, and occurred with concomitant oxidation of sulfite to sulfate. In the dark, both ethane formation and sulfite oxidation were inhibited. Ethane formation was stimulated by ferric or ferrous ions and inhibited by ethylenediamine tetraacetate. The photosynthetic electron transport modulators, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and phenazine methosulfate, inhibited both sulfite oxidation and ethane formation. Methyl viologen greatly stimulated ethane formation, but had little effect on sulfite oxidation. Methyl viologen, in the absence of sulfite, caused only a small amount of ethane formation in comparison to that produced with sulfite alone. Sulfite oxidation and ethane formation were effectively inhibited by the radical scavengers, 1,2-dihydroxybenzene-3,5-disulfonic acid and ascorbate. Ethanol, a hydroxyl radical scavenger, inhibited ethane formation only to a small degree; formate, which converts hydroxyl radical to superoxide radical, caused a small stimulation in both sulfite oxidation and ethane formation. Superoxide dismutase inhibited ethane formation by 50% when added at a concentration equivalent to that of the endogenous activity. Singlet oxygen did not appear to play a role in ethane formation, inasmuch as the singlet oxygen scavengers, sodium azide and 1,4-diazobicyclo-[2,2,2]-octane, were not inhibitory. These data are consistent with the view that O_2 is reduced by the photosynthetic electron transport system to superoxide anion, which in turn initiates the free radical oxidation of sulfite, and the free radicals produced during sulfite oxidation were responsible for the peroxidation of membrane lipids, resulting in the formation of ethane.

Sulfur dioxide is a major air pollutant causing damage to plants. The increasing demand for the use of coal for power generation may lead to an increase in SO_2 pollution (28). Various physiologic parameters are affected in plants exposed to SO_2. These include inhibition of photosynthesis and growth rate which can occur without visible injury (13). One of the first ultrastructural changes observed in plants exposed to SO_2 is damage to chloroplast membranes (9, 13, 34), resulting in a loss of membrane integrity, which is vital to all processes in the plant. Proteins are susceptible to attack by sulfite (30) which could lead to altered membrane structure and function. Recent work from our laboratory (20) has shown that sulfite can induce the *in vitro* peroxidation of linoleic and linolenic acid which could lead to the alteration of membranes. Inasmuch as these two fatty acids comprise approximately 75% of those found in chloroplast membranes, peroxidation of these fatty acids may be an important factor contributing to damage *in vivo*.

Sulfite can undergo very rapid oxidation to sulfate through a free radical mechanism which predominates at low concentrations (1, 30). Free radicals produced during the oxidation of sulfite have been reported to effect the *in vitro* destruction of methionine and tryptophan (35, 36), indole-3-acetic acid (37), Chl (26), β-carotene (25), and oxidized NADH and NADPH (33). Also the peroxidation of linolenic and linoleic acids has been attributed to free radicals produced during sulfite oxidation (20).

Because chloroplasts are rich in linoleic and linolenic acid and appear to be an early site of damage by SO_2, we have studied whether sulfite could induce lipid peroxidation in chloroplasts via free radical mechanisms.

MATERIALS AND METHODS

Chloroplasts were isolated from spinach (*Spinacia oleracea* L.) obtained from a local grower or from a market. Leaves were passed through a juice extractor (Acme Supreme) along with isolation medium containing 50 mM phosphate buffer (pH 7.8), 0.33 M sorbitol and 2 mM $MgCl_2$ at 0°C. Chloroplasts were passed through Miracloth and then centrifuged 2 min at 1,700*g*. The pellet was resuspended in incubation medium (same as the isolation medium except that 100 mM glycylglycine [pH 7.8] was used instead of phosphate buffer) and then layered on top of Percoll medium (40% [v/v] Percoll in incubation media). This was centrifuged in a swinging bucket rotor for 5 min at 3,000*g*. Broken chloroplasts remained at the buffer-Percoll interface while intact chloroplasts penetrated the Percoll. Intactness was 80% or greater as estimated by the ferricyanide method (19). Freeze-treated chloroplasts were prepared by placing broken chloroplasts at −10°C overnight.

Lipid peroxidation from chloroplasts was determined by measuring ethane formation (6, 8). A standard reaction mixture containing chloroplasts (400 μg Chl) and 0.1 μmol $FeCl_3$ in 1 ml incubation medium was incubated in a 10-ml Erlenmeyer flask, which was sealed with a serum stopper and gently shaken at 25°C over a 15 w cool-white fluorescent lamp which provided 200 $\mu E \cdot m^{-2} \cdot s^{-1}$ of illumination. Seven μl of solution containing 1.4 μmol Na_2SO_3 plus 0.7 nmol EDTA or 1.4 μmol Na_2SO_4 plus 0.7 nmol EDTA in controls were added every 3 min using a syringe with hypodermic needle. EDTA was included in the sulfite solution to prevent autooxidation. At various times, the gas headspace was sampled and injected into a gas chromatograph equipped with an alumina column for ethane measurement.

Sulfite oxidation was measured both by sulfite determination using the pararosaniline method (29) and by measuring O_2 uptake. O_2 uptake was determined in a 3 ml reaction mixture containing 200 μg Chl with a Clark O_2 electrode under darkness or under 1,500 $\mu E \cdot m^{-2} \cdot s^{-1}$ of red illumination.

[1] Supported by a research grant from the California State Air Resources Board (A1-070-33).

[2] Present address: ASEAN—Postharvest Horticulture Training and Research Center, University of the Philippines at Los Baños, College, Laguna, Philippines.

RESULTS

Ethane, derived from the decomposition of the 16-hydroperoxide of linolenic acid, has been used in many systems as a measure of lipid peroxidation (6, 8, 27). In broken and intact chloroplasts sulfite greatly stimulated ethane production compared with sulfate (sulfate was always added to controls) (Table I). Sulfite caused approximately a 10-fold increase in ethane production in intact chloroplasts as compared to the sulfate control. The effect of sulfite, however, was even more marked in broken chloroplasts. Ethane production from freeze-treated broken chloroplasts produced the greatest amounts of ethane (Table I).

Differences in sulfite-induced ethane formation were consistently observed with chloroplasts isolated from spinach grown in different seasons. Chloroplasts from winter spinach produced more ethane (800–900 pmol/h) than those from spring spinach (300–400 pmol/h). The effects of certain metals were examined to determine if ethane formation could be increased in chloroplasts from spring spinach. Ethane formation was enhanced by the addition of 100 μM $FeCl_3$ and greatly reduced by 1 mM EDTA (Fig. 1). $FeSO_4$ was as effective as $FeCl_3$, whereas $CuCl_2$ (100 μM) had no effect upon ethane production. Whether an inhibitor was present or lower concentrations of stimulators (such as metal ions) were present in spring compared to winter spinach was not examined. $FeCl_3$ was routinely added to the incubation media. When $FeCl_3$ was deleted, less ethane was formed, although no qualitative changes were observed. In some systems (17), cupric or cuprous ion modifies the ratio of ethylene to ethane produced from linolenic acid, but in our system it had little effect upon this ratio. Ethylene production was usually only 3% or less of the ethane production.

Light was very important for sulfite-induced ethane formation. In the dark only about 10 to 20 pmol ethane was produced after 1 h in the presence or absence of sulfite with or without $FeCl_3$ (Fig. 1). $MnCl_2$ was not added to the isolation or incubation medium because it promoted ethane production in the dark with sulfite. In several systems Mn^{2+} has been used to initiate the free radical oxidation of sulfite (26, 35, 36, 37).

Sulfite oxidation, measured by sulfite loss, occurred throughout the incubation period although complete oxidation did not occur (Fig. 2). The amount of sulfite added, 1.4 μmol every 3 min, was necessary because a 25% reduction in sulfite concentration caused a 70% reduction in ethane formation. One explanation for these results is that a portion of the sulfite reacted with some chloroplast component forming a complex or adduct which was stable against oxidation, yet it would react with pararosaniline reagent in the sulfite assay. In the dark, little sulfite was oxidized with or without

Table I. *Ethane Formation from Broken, Intact, and Freeze-Treated Chloroplasts*

Standard reaction conditions were used and incubation time was 1 h in the light.

Chloroplasts	Ethane	
	pmol	%
Broken		
$+SO_3^{2-}$	491	100
$+SO_4^{2-}$	20	4
Intact		
$+SO_3^{2-}$	46	9
$+SO_4^{2-}$	3	1
Freeze-treated		
$+SO_3^{2-}$	2,995	610
$+SO_4^{2-}$	65	13

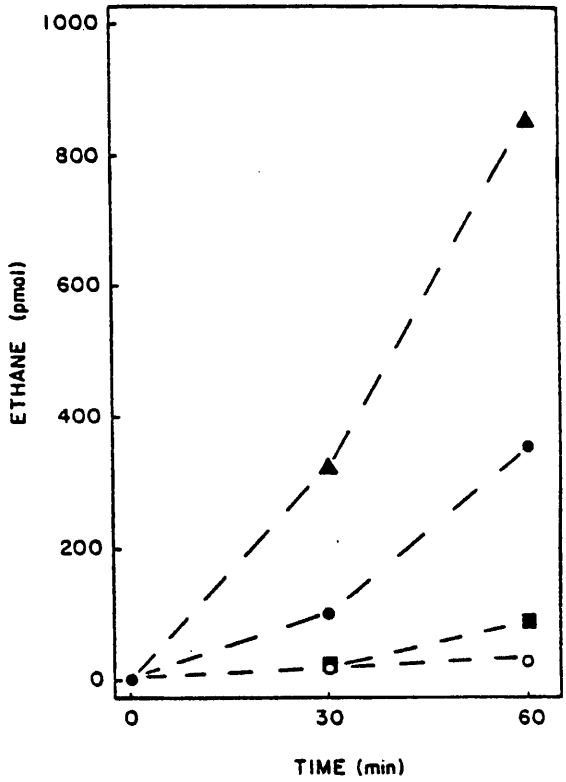

FIG. 1. Ethane production from broken chloroplasts incubated in the light with sulfite (●), sulfite + $FeCl_3$ (100 μM) (▲), sulfite + EDTA (1 mM) (■), or sulfate (○). In the dark, ethane production was less than 20 pmol·h^{-1} in all treatments.

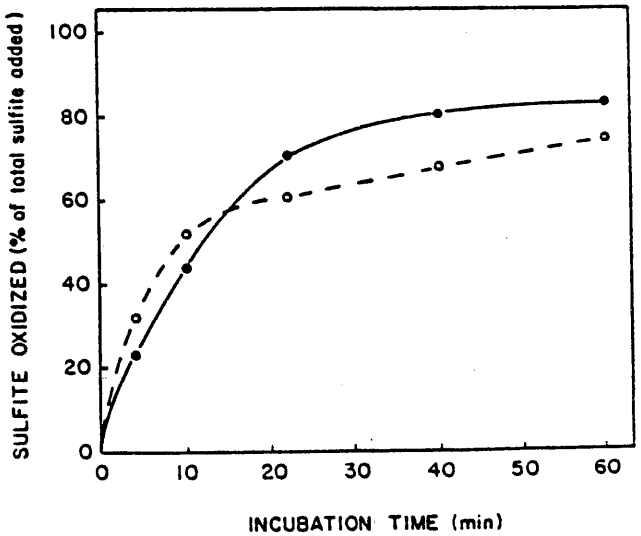

FIG. 2. Sulfite oxidation measured as sulfite loss by the pararosaniline assay during incubation in the light (200 μE·m^{-1}) with sulfite (●), or sulfite plus MV (100 μ_m) (○). In the dark, ethane production was less than 20 pmol·h^{-1} in all treatments.

100 μM $FeCl_3$ (data not shown). When sulfite oxidation was measured using the O_2 electrode, the O_2 uptake rate in dark was approximately 24 nmol/min, whereas that in the light was approximately 240 nmol/min, measured in a 3-ml volume. These rates are similar to those obtained by Asada and Kiso (3) examining sulfite oxidation in chloroplasts.

Inasmuch as light was necessary for sulfite-induced ethane

production as well as sulfite oxidation, the effect of various photosynthetic electron transport modulators was examined (Table II). DCMU, which inhibits photosynthetic electron transport, as well as PMS,[3] which promotes cyclic electron flow, inhibited both ethane production and sulfite oxidation (measured as O_2 uptake). These results demonstrate the dependence of sulfite oxidation and ethane formation upon photosynthetic electron transport and noncyclic electron flow. MV (paraquat), which facilitates the reduction of O_2 on the reducing side of PSI to form O_2^- (7), greatly stimulated ethane production in the presence of sulfite, but caused a comparatively small amount of ethane formation in the absence of sulfite. Although we measured sulfite oxidation in the presence of MV with the oxygen electrode, this is complicated because oxygen uptake occurs with MV in the light in the absence of sulfite. Therefore, we determined sulfite oxidation in the presence of MV using the pararosaniline assay, under identical conditions as for ethane formation. Contrary to our expectation, MV had only a small effect upon sulfite oxidation (Fig. 2) in comparison with its effect upon ethane formation in the presence of sulfite (Table II).

The participation of free radicals was implicated by the effective inhibition of both sulfite oxidation and ethane formation by the radical scavengers, tiron and ascorbate (Table III). Tiron has been reported to be a specific scavenger for O_2^- (12), but recent evidence indicates that it also effectively scavenges hydroxyl radical (5). Ascorbate can likewise react with O_2^- and hydroxyl radical (14). Ethanol, a hydroxyl radical scavenger, caused a small amount of inhibition of ethane formation; formate, which converts hydroxyl radical to O_2^- (4), caused a small stimulation of ethane formation and sulfite oxidation. The participation of hydroxyl radical is further suggested from preliminary observations that when mannitol was used in place of sorbitol as osmoticum, an inhibition of ethane production was observed. Mannitol is a hydroxyl radical scavenger and approximately twice as effective as sorbitol in this respect (3). Glycylglycine was specifically chosen as the buffer since Tricine and Hepes inhibited ethane formation. Asada and Kiso (3) also reported that Tris and Tricine inhibited sulfite oxidation in illuminated chloroplasts.

The close interrelation between sulfite oxidation and ethane formation and their dependence upon photosynthetic electron transport is demonstrated in the above results. In the absence of photosynthetic electron transport in the dark (Fig. 1) or upon its inhibition with DCMU and PMS (Table II), sulfite oxidation and ethane formation were inhibited. Also, the radical scavengers tiron and ascorbate effectively inhibited both sulfite oxidation and ethane formation.

Inasmuch as singlet oxygen has been reported to be involved in the peroxidation of chloroplast lipids (31), we examined the effect of DABCO and sodium azide (Table III). DABCO has been used as a singlet oxygen scavenger, although recent evidence indicates it also can serve as an effective radical scavenger (24). Sodium azide is considered an effective quencher of singlet oxygen with a rate constant of 2.2×10^8 $M^{-1} \cdot s^{-1}$ at 0.5 mM (15). Results from these two compounds (Table III) do not indicate the participation of singlet oxygen in the production of ethane in our system. Chloroplasts contain both the Cu, Zn, and the Mn forms of superoxide dismutase (2). The stimulation of ethane formation and sulfite oxidation with azide and cyanide (Table III) may result from their inhibition of Cu, Zn-superoxide dismutase thereby increasing the concentration of O_2^-. Additionally, azide and cyanide could stimulate sulfite oxidation by complexing with metals which might stimulate radical-mediated reactions (21).

The specific involvement of O_2^- was indicated by inhibition of ethane formation with superoxide dismutase (Table IV). The amount of superoxide dismutase added was equivalent to the amount of endogenous enzyme activity of intact chloroplasts as determined by the xanthine:xanthine oxidase assay. The lack of complete inhibition with superoxide dismutase suggests that either another radical in addition to O_2^- is involved or the site of O_2^- formation, presumably on the thylakoid membranes, is not easily accessible by the exogenously added superoxide dismutase; in contrast, this site might be more accessible to a much smaller molecule like tiron which renders it a more effective inhibitor. Although the small inhibition by catalase indicates that H_2O_2 was participating in ethane formation, in intact but not broken chloroplasts, an effective H_2O_2 scavenging system exists (23) which presumably would greatly reduce the participation of H_2O_2 in intact leaves. Since BSA had no effect upon ethane formation, the effects of superoxide dismutase and catalase were catalytic and not simply a nonspecific protein effect.

In most of these experiments $FeCl_3$ was present along with a small amount of EDTA (0.7 nmol added every 3 min with SO_3^{2-}).

Table II. *Effect of DCMU, Phenazine Methosulfate, and Methyl Viologen upon Ethane Formation and Sulfite Oxidation from Broken Chloroplasts*

Incubation time for ethane formation was 1 h in the light. The rate of O_2 uptake was determined from the linear portion of the uptake curve and the small dark rate was subtracted from the light rate.

Addition	Ethane	O_2 Uptake
	%	
Sulfite	100[a]	100[b]
Sulfite + DCMU, 10 μM	17	12
Sulfite + PMS, 20 μM	20	18
Sulfite + MV, 100 μM	685	
Sulfate + MV, 100 μM	8	

[a] Ethane production was 284 pmol.
[b] Rate of O_2 uptake was 195 nmol·min^{-1}.

Table III. *Effect of Various Compounds on Ethane Formation and Sulfite Oxidation in Broken Chloroplasts*

Reaction conditions as for Table II.

Addition	Ethane	O_2 Uptake
	%	
None	100[a]	100[b]
Tiron, 1 mM	3	7
Ascorbate, 3 mM	2	5
Ethanol, 3%	83	
Ethanol, 1%	100	
Formate, 10 mM	133	126
DABCO, 10 mM	110	102
Azide, 10 mM	160	205
Cyanide, 1 mM	135	180

[a] Ethane production was 460 pmol.
[b] Rate of O_2 uptake was 240 nmol·min^{-1}.

Table IV. *Effect of Superoxide Dismutase, Catalase, and BSA on Ethane Formation in Broken Chloroplasts*

Incubation time was 1 h in the light. Chloroplasts containing 300 μg Chl/ml were used.

Addition	Ethane
	%
None	100[a]
Superoxide dismutase (30 μg; 60 units)	49
Catalase (30 μg; 1,100 units)	77
BSA (30 μg)	107

[a] Ethane production was 518 pmol.

[3] Abbreviations: PMS, phenazine methosulfate; MV, methyl viologen; DABCO, 1,4-diazobicyclo-[2,2,2]-octane; tiron, 1,2-dihydroxybenzene-3,5-disulfonic acid.

Fe-EDTA can stimulate free radical reactions in some systems (21). In our chloroplast system, however, comparable amounts of ethane were formed in the presence or absence of $FeCl_3$ and EDTA. The amount of inhibition of ethane formation by superoxide dismutase and stimulation by formate was not altered when $FeCl_3$ and EDTA were excluded. This indicates our results are not dependent upon an Fe-EDTA complex, but does not exclude the involvement of an endogenous metal complex.

DISCUSSION

The aerobic oxidation of sulfite can be initiated by UV light (30), metals (1, 35), photosensitized dyes (26) and enzymic reactions (10), all of which produce free radicals. The superoxide radical appears to be the radical responsible for initiation in some of these cases (35), and a scheme for this reaction has been proposed (1, 35).

$$O_2^-\cdot + SO_3^{2-} + 2H^+ \rightarrow SO_3^-\cdot + 2OH\cdot \quad (1)$$
$$SO_3^-\cdot + O_2 \rightarrow SO_3 + O_2^-\cdot \quad (2)$$
$$SO_3^{2-} + OH\cdot + H^+ \rightarrow SO_3^-\cdot + H_2O \quad (3)$$
$$SO_3^-\cdot + OH\cdot + H^+ \rightarrow SO_3 + H_2O \quad (4)$$
$$2SO_3^-\cdot \rightarrow SO_3 + SO_3^{2-} \quad (5)$$
$$SO_3 + H_2O \rightarrow SO_4^{2-} + 2H^+ \quad (6)$$

Sulfite oxidation is maintained by the propagation reactions (equations 1, 2, 3) with the production of $O_2^-\cdot$, $OH\cdot$ and $SO_3^-\cdot$. The termination reactions (equations 4, 5, 6) lead to sulfate formation.

Asada and Kiso (3) reported that photosynthetic electron transport of illuminated chloroplasts initiated the aerobic free radical oxidation of sulfite. Experimental evidence indicates that O_2 serves as an electron acceptor on the reducing side of PSI forming O_2^- (2, 11) which in turn initiates sulfite oxidation. Our results are similar to those reported by Asada and Kiso (3), showing the dependence of sulfite oxidation on photosynthetic electron transport. They observed inhibition of sulfite oxidation by DCMU and radical scavengers as we observed (Tables II and III). Our results link photosynthetic electron transport-initiated sulfite oxidation to the peroxidation of membrane lipids in chloroplasts, by which SO_2 damage to plants might be mediated. In each experiment where sulfite oxidation was either inhibited or stimulated, ethane formation responded likewise, except for the experiment with MV. MV increased ethane formation 6- to 7-fold over that caused by sulfite alone (Table II) but had little effect upon sulfite oxidation (Fig. 2). One possible explanation is that MV cation interacts with sulfite or products of sulfite oxidation producing a very reactive species which greatly enhances ethane formation without affecting sulfite oxidation.

Sulfite-induced lipid peroxidation has been reported in corn oil emulsions (16) and emulsions of linoleic acid and linolenic acid (20). This peroxidation of linoleic and linolenic acids appeared to proceed by a free radical reaction according to the following scheme where LH represents linoleic or linolenic acid (20):

$$SO_3^-\cdot + LH \rightarrow \cdot LHSO_3^- \quad (7)$$
$$\cdot LHSO_3^- + HSO_3^- \rightarrow LH_2SO_3 + SO_3^-\cdot \quad (8)$$
$$SO_3^-\cdot + LH \rightarrow \cdot L + HSO_3^- \quad (9)$$
$$\cdot L + O_2 \rightarrow LOO\cdot \quad (10)$$
$$LOO\cdot + LH \rightarrow LOOH + \cdot L \quad (11)$$

The authors suggested that $SO_3^-\cdot$, which could result from equation 1 or 3, was the important radical which mediated the propagation steps via addition (equation 7) and hydrogen abstraction (equation 9). Hydrogen abstraction from polyunsaturated fatty acids is considered to be one of the primary steps in the free radical-mediated peroxidation of polyunsaturated fatty acids (22). Similarly, hydrogen abstraction from NADH by $SO_3^-\cdot$ has been demonstrated during the sulfite-mediated oxidation of NADH to NAD (33). Based on this information and our results, we propose that of the radicals formed during the O_2^- initiated sulfite oxidation (equations 1, 2, 3) $SO_3^-\cdot$ is the primary radical causing the peroxidation of chloroplast lipids. $OH\cdot$ appears to play only a minor role since ethanol and formate had small effects upon ethane formation (Table III). O_2^- alone does not appear to be important in ethane formation since MV, which facilitates O_2^- formation without sulfite, caused a relatively small amount of ethane formation (8%) as compared with that caused by sulfite (Table II).

However, we cannot exclude the possibility that lipid peroxidation results from an interaction of two or more radicals rather than only one radical. An alternative explanation for the enhanced ethane production from sulfite and MV is that this synergism results from an interaction between O_2^-, at an increased concentration resulting from MV, and other radicals from sulfite oxidation. Kong and Davison (18) have shown that interactions between oxy radicals could lead to greater amounts of membrane permeability in erythrocyte ghosts than was expected from the summed effects of the individual radicals.

An outline of our results is presented in Figure 3. Photosynthetic

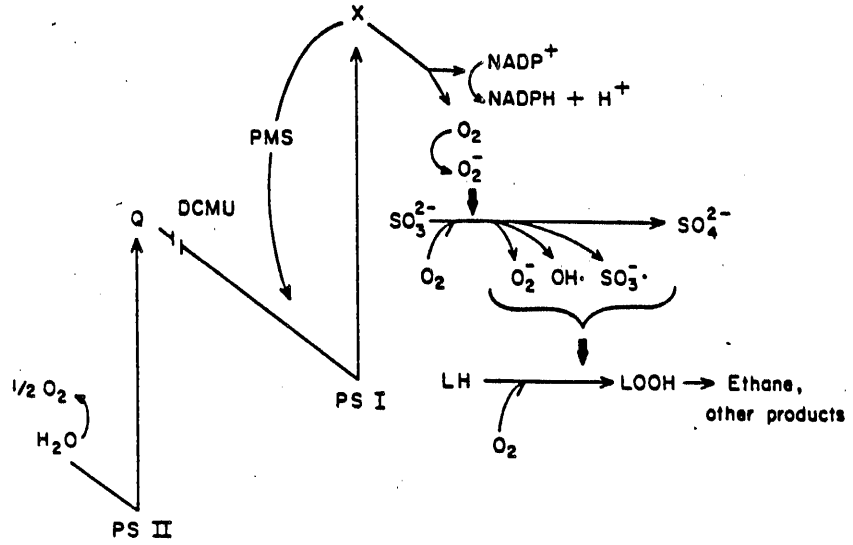

FIG. 3. Proposed scheme for sulfite-induced ethane formation in illuminated chloroplasts. (♦), an initiation reaction.

electron transport provides electrons to reduce O_2 to O_2^- which initiates sulfite oxidation. Radicals produced from sulfite oxidation then lead to the peroxidation of membrane lipids resulting in the formation of ethane.

Further work is needed to determine whether this free radical mechanism of sulfite-induced lipid peroxidation plays an important role in the *in vivo* damage to plants exposed to SO_2. However, regardless of the specific mechanism, there is evidence from *in vivo* experiments implicating free radicals, specifically O_2^-, in SO_2 phytotoxicity. Tanaka and Sugahara (32) have reported that young poplar leaves contained more superoxide dismutase activity and were more resistant to SO_2 injury than old leaves. Also, they observed that low levels of SO_2 could induce superoxide dismutase activity in leaves and these leaves were subsequently more resistant to injury by high levels of SO_2 than leaves without the prefumigation.

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